NATURAL INHIBITORS OF ENZYMATIC BROWNING

By

RUHIYE YORUK

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2003

Copyright 2003

by

Ruhiye Yoruk

This work is dedicated to those in the world who care most: My parents, Munevver and Nizamettin Yoruk; and my sister and brother, Seviye and Fahri. Your years of selfless love and unwavering support, near and afar, always found a way to reach me. It is through you that I am able to succeed in this endeavor. Thank you.

ACKNOWLEDGMENTS

I would like to take this opportunity to thank my advisor, Dr. Marshall, for his enthusiasm and continued support throughout my graduate studies here at the University of Florida. I also appreciate and am indebted to him for his personal guidance and friendship during my graduate program. He has always impressed me with his ability to adapt publicly to any audience. I had tremendous scientific and decisive professional experiences at his laboratory. For this, I am truly grateful.

I would like to express my sincere appreciation to Dr. Balaban, Dr. Hogsette, Dr. Gregory, and Dr. Preston for serving on my supervisory committee. I extend special thanks to Dr. Balaban and Dr. Hogsette for their personal friendship and professional guidance. I would like to thank Dr. Butler for his guidance in rearing houseflies at the beginning of this research and for his continued encouragement. I extend special thanks to Dr. Gregory, and Dr. Preston for their help in making this project a positive experience. I would also like to thank Dr. Gregory, Dr. Preston, Dr. Sims, Dr. Talcott, and Dr. Kristinsson for their invaluable comments and suggestions on the manuscripts.

I would like to remember Diana, who passed away suddenly in 2001, and thank her for assisting me in the housefly production and for making an enjoyable work environment in the Department of Entomology. I extend special thanks to the personnel of the U. S. Department of Agriculture in Gainesville for their assistance in the production of housefly pupae. I appreciate and am indebted to Mr. Hughes for keeping my computer running and for the free strawberries and oranges.

I would also like to take this opportunity to thank my parents, Munevver and Nizamettin Yoruk (the best advocate one could ever have) for always believing in me. Their unwavering support and caring provides the drive to achieve my goals. I also appreciate and am indebted to my brother, Fahri, for his undying encouragement. His selfless love and eternal support always found a way to reach me from thousands of miles away. To my sister, Seviye, I owe all my success. She is driven by personal excellence, and is my best friend and role model in strength and courage. I would like to thank God for giving me the strength and aptitude to withstand the tribulations of this endeavor.

This research was supported in part by funding from Agro-Industries and Post-Harvest Management Service, Agricultural Support Systems Division in the Food and Agricultural Organization of the United Nations. I would also like to thank the U. S. Department of Agriculture, Center for Medical, Agricultural, and Veterinary Entomology in Gainesville for providing housefly pupae.

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS iv

LIST OF TABLES ix

LIST OF FIGURES x

ABSTRACT xii

INTRODUCTION 1

LITERATURE REVIEW 5

Importance of Enzymatic Browning in the Food industry 5
Problem 5
Biochemical Foundation of the Problem 6
Polyphenol Oxidase (PPO) 9
Molecular Mechanism of Action 9
Plant PPO 12
General Properties of Apple and Banana PPO 17
Substrate specificity 18
Effect of pH and temperature 20
Insect PPO 23
Inhibition/Prevention of Enzymatic Browning 27
Oxalic Acid 32

MODE OF INHIBITION OF POLYPHENOL OXIDASE BY OXALIC ACID 34

Introduction 34
Materials and Methods 36
Preparation of PPO 36
Preparation of Oxalic Acid Solutions 37
Assay of PPO Activity and Inhibition 37
Preincubation of PPO with Oxalic Acid 38
Treatment of Oxalic Acid with Cupric Acetate 38
Restoration of Enzymatic Activity of Oxalic Acid-Treated PPO 39
Scanning Studies 39
Inhibitory Effect of Dicarboxylic Acids 40
Results and Discussion 40
The pH Activity Profile of PPO 40

Concentration- and pH-dependent Inhibitory Effect of Oxalic Acid 40
Effect of Preincubation on Inhibition by Oxalic Acid 43
Inhibition Kinetics 45
Chelating Effect of Oxalic Acid on the Active-Site Copper 46
Scanning Studies 50
Comparison of Inhibitory Effectiveness of Dicarboxylic Acids 53

MACHINE-VISION ANALYSIS OF ANTIBROWNING POTENCY FOR OXALIC ACID: A COMPARATIVE INVESTIGATION ON BANANA AND APPLE 55

Introduction 55
Materials and Methods 57
Preparation of Fruit Slices and Their Treatments with Oxalic Acid 57
Comparison of Antibrowning Effectiveness of Dicarboxylic Acids 58
Color Data Analysis 58
Color Spectra 59
Statistical Analysis 59
Determination of pH and Oxalic Acid Concentration in the Treated Tissues 59
Results and Discussion 60
Visual Observation 60
Effect of Oxalic Acid and Storage Time 62
Changes in Color Spectra 69
The pH and Oxalic Acid Concentration 74
Comparison of Antibrowning Effectiveness of Dicarboxylic Acids 76

INVESTIGATIONS ON POTENTIAL OF OXALIC AND ASCORBIC ACIDS TO CONTROL BROWNING OF MINIMALLY PROCESSED APPLE AND BANANA USING MACHINE VISION 81

Introduction 81
Materials and Methods 82
Preparation of Fruit Slices and Their Treatments with Oxalic and Ascorbic Acids 82
Color Data Analysis 83
Statistical Analysis 84
Determination of Oxalic Acid Concentration in the Treated Tissues 84
Results and Discussion 84
Effect of Ascorbic Acid on Browning Inhibition 84
Antibrowning Potency of Oxalic Acid in Conjunction with Ascorbic Acid on Apple Slices 85
Antibrowning Potency of Oxalic Acid in Conjunction with Ascorbic Acid on Banana Slices 98

APPLE POLYPHENOL OXIDASE INHIBITOR(S) FROM COMMON HOUSE FLY (*Musca domestica* L.) 102

Introduction 102

Materials and Methods 104
House Fly Pupae 104
Preparation of Inhibitor Extract 104
Preparation of Apple PPO 106
Assay of PPO Activity and Inhibition 108
The pH Optimum of Apple PPO 108
Effect of Heating and Freezing and Thawing on Inhibitor(s) 109
The pH Stability of Inhibitor(s) 109
Gamma Irradiation 109
Ultrafiltration 110
Protein Determination 110
Preliminary Purification Studies 110
Acetone precipitation 110
Ammonium sulfate precipitation and column chromatography 111
Results and Discussion 112
Optimization of pH Conditions for PPO Activity and Inhibition 112
Inhibitor Activity of the House Fly During Metamorphosis 115
Characterization of Crude Inhibitor(s) From House Fly Pupae 117
Effect of inhibitor concentration 117
Temperature stability 118
The pH stability 119
Gamma irradiation 120
Ultrafiltration 120
Preliminary Studies on Purification of House Fly Inhibitors 122
Solvent fractionation 122
Ammonium sulfate fractionation and column chromatography 122

CONCLUSIONS 125

LIST OF REFERENCES 127

BIOGRAPHICAL SKETCH 141

LIST OF TABLES

Table page

2-1. Relative substrate specificity of PPOs from apple and banana 19

2-2. Estimated oxalic acid content of some common foods of plant origin 33

3-1. Comparison of inhibitory effect of dicarboxylic acids on PPO activity.....54

4-1. Change in pH with oxalic acid concentration in the treated tissues 74

6-1. Heating effect on inhibitor activity.....118

6-2. Ultrafiltration of inhibitor preparation.....121

LIST OF FIGURES

Figure page

2-1. Enzymatic and subsequent nonenzymatic condensation processes for browning.....7

2-2. Proposed monophenol and diphenol oxidation pathways of *Neurospora crassa* PPO.....11

2-3. Enzymatic oxidation of the phenolic amino acid tyrosine to melanin 13

2-4. Mechanism of prevention of enzymatic browning by ascorbic acid 29

3-1. Effect of pH on the catalytic activity of PPO 41

3-2. Effect of pH and oxalic acid concentration on the inhibition of PPO activity 41

3-3. Effect of preincubation on the inhibition by oxalic acid for PPO at different pH values.....44

3-4. Kinetic determination of mode of inhibition 45

3-5. Effect of cupric acetate treated-oxalic acid on PPO activity 47

3-6. Effect of added copper, at a constant enzyme concentration, on the restoration of enzymatic activity to oxalic acid-treated PPO 49

3-7. Effect of oxalic acid on the absorption spectrum of the final product(s) of PPO-catechol reaction 50

3-8. Incubation of oxalic acid with the final product(s) of PPO-catechol reaction 52

4-1. Video images of oxalic acid treated banana and apple slices during storage.....61

4-2. Changes in browning ($L_0^* - L_t^*$) on cut surfaces of banana as a function of oxalic acid concentration and storage time 63

4-3. Browning indices on cut surfaces of oxalic acid treated banana.....65

4-4. Changes in browning ($L_0^* - L_t^*$) on cut surfaces of apple as a function of oxalic acid concentration and storage time 67

4-5. Browning indices on cut surfaces of oxalic acid treated apple.....68

4-6. Temporal changes in color spectra on cut surfaces of oxalic acid treated banana 70

4-7. Temporal changes in color spectra on cut surfaces of oxalic acid treated apple.....72

4-8. Comparison of antibrowning effectiveness of dicarboxylic acids on banana and apple slices 77

4-9. Changes in color spectra during browning of apple treated with dicarboxylic acids.79

5-1. Effect of ascorbic acid on browning inhibition of apple and banana slices 86

5-2. Effect of oxalic acid in conjunction with ascorbic acid on browning inhibition of apple slices 87

5-3. Browning indices on cut surfaces of oxalic and ascorbic acids treated apple.....89

5-4. Temporal changes in color spectra on cut surfaces of oxalic acid and ascorbic acid treated apple 92

5-5. Effect of oxalic acid in conjunction with ascorbic acid on browning inhibition of banana slices.....99

5-6. Browning indices on cut surfaces of oxalic acid and ascorbic acid treated banana .100

6-1. Pictures of some laboratory materials for production of house fly 105

6-2. Autoseparation of house fly larvae from culture medium using Berlese funnel 107

6-3. The pH optimum of apple PPO. 113

6-4. Inhibition of apple PPO activity by inhibitor(s) from house fly pupae 114

6-5. Optimum pH for PPO inhibition by pupal extract.....114

6-6. Life cycle of house fly 116

6-7. Inhibition by house fly extract on apple PPO through its developmental stages 116

6-8. Effect of inhibitor concentration on PPO activity. 117

6-9. The pH stability of inhibitor(s).....119

6-10. Elution profile of inhibitor activity on Sephadex G-25 123

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

NATURAL INHIBITORS OF ENZYMATIC BROWNING

By

Ruhiye Yoruk

December 2003

Chair: Maurice R Marshall
Department: Food Science and Human Nutrition

Millions of dollars in crop losses occur yearly as a result of enzymatic browning. This oxidative browning reaction caused by polyphenol oxidase (PPO) results in deterioration of food quality and limits production of specialty products like sliced fruits. Prevention of unfavorable browning would have enormous economic benefits to the food industry and consumers; thus, studies were conducted to characterize two natural browning inhibitors: oxalic acid and inhibitor(s) isolated from common house fly (*Musca domestica* L.).

The mode of inhibition for oxalic acid on PPO was investigated. The extent of inhibition was influenced by oxalic acid concentration and also by pH changes. Inhibition of PPO by oxalic acid was due to its binding with copper to form an inactive complex, and the inhibition was characterized as noncompetitive. Oxalic acid was a more potent inhibitor of PPO compared with other structurally related acids. The relative antibrowning potency of oxalic acid on fresh-cut surfaces of bananas and apples was investigated using a machine-vision system. Degree of browning inhibition was evaluated

based on oxalic acid concentration and storage time. Browning inhibition was most prominent on banana and apple slices treated with oxalic acid solutions at concentrations of 60 and 5 mM, respectively. Combining oxalic acid with ascorbic acid synergistically enhanced browning inhibition for apple slices, but was not effective for bananas. Apple browning was suppressed with a solution containing 0.5% ascorbic acid and oxalic acid in the range of 1.5 to 5 mM. Average residual oxalic acid levels in the tissues for an effective antibrowning activity were measured.

Additionally, a new type of inhibitor(s) was discovered from house fly extracts. The inhibitor(s) isolated by buffer extraction, heat treatment, and dialysis from house fly pupae inhibited the activity of apple PPO up to 90% at pH values above 5.0. Inhibitor(s) was further characterized by employing heat, freezing and thawing, irradiation, pH adjustment, and ultrafiltration studies. Potential PPO inhibitor(s) was stable to heating at 100°C for 1 h, repeated freezing and thawing, and irradiation. Inhibitor(s) was most stable near pH 5.0 and least stable at alkaline pH. The PPO inhibition profile of the house fly during metamorphosis was also evaluated.

CHAPTER 1
INTRODUCTION

Enzymatic browning, associated with polyphenol oxidase (PPO; EC 1.10.3.1), causes millions of dollars in damage yearly to fruits and vegetables (Whitaker and Lee 1995; Yoruk and Marshall 2003). PPO catalyzes the hydroxylation of monophenols and the oxidation of *o*-diphenols into *o*-quinones with oxygen as the primary oxidant (Whitaker 1994; Lerch 1995; Yoruk and Marshall 2003). The highly reactive quinones usually undergo further nonenzymatic condensation reactions leading to the generation of unpleasant dark brown melanins (Mathew and Parpia 1971; Sapers 1993). Quality of many fruits and vegetables susceptible to this biochemical reaction diminishes rapidly due to unpleasant discoloration and off-flavor generation (Vamos-Vigyazo 1981; Zawistowski and others 1991; Yoruk and Marshall 2003).

Bananas and apples are popular fruits in the world, with world production exceeding 35 and 85 million tons/yr, respectively (Nicolas and others 1994; Becker and others 2000). They are prone to enzymatic browning during handling and processing, and the fruit industry is interested in methods to prevent browning of minimally processed crops. Browning has a significant impact on the quality of bananas, apples, and their products as it results in alteration of color, flavor, texture, and nutritional value (Nicolas and others 1994; Gooding and others 2001). Therefore, it is necessary to maintain the original color and quality of the fresh fruits during processing and in the market. Sulfiting agents were used broadly in the fruit and vegetable industry as antibrowning agents because of their effectiveness and low cost (Eskin 1990). However, due to safety

concerns, their use in fresh fruit and vegetables was banned by the Food and Drug Administration (Sapers 1993). There is interest in effective, natural, and economical antibrowning agents due to consumers' and the industry's demand for natural ingredients. Hence, studies were conducted to characterize two natural PPO inhibitors, oxalic acid and inhibitors isolated from house fly (*Musca domestica* L.).

There are few reports on the inhibition of PPO by oxalic acid. Studies by Sato (1980a) demonstrated oxalic acid as a naturally occurring inhibitor of PPO in spinach. Ferrar and Walker (1993) reported that oxalic acid produced by the phytopathogen *Sclerotinia sclerotiorum* inhibited PPO activity in infected apples and bean pods. Oxalic acid inhibited the activities of PPOs from sunflower (Marciano and others 1983), sweet cherry (Pifferi and others 1974), and mushroom (Yong and others 1990; Son and others 2000a). There is limited information on the inhibition mechanism of oxalic acid on PPO. Thus, the first objective of this study was to define the mode of oxalic acid inhibition on PPO; and the second objective was to compare its inhibitory effectiveness to some other structurally related compounds including malonic, glutaric, and succinic acids.

Although it is not an approved food additive, oxalic acid is a natural component of a large number of foods from plant origin such as asparagus, broccoli, Brussel sprouts, carrot, garlic, lettuce, onion, parsley, pea, potato, purslane, radish, spinach, tomato, turnip, rhubarb, and several wild edible plants (Sato 1980a; USDA 1984; Guil and others 1996; Son and others 2000b). There is little, if any, documentation on the applicability of oxalic acid in prevention of browning in minimally processed banana. There are few reports on antibrowning effect of oxalic acid on Liberty apples (Son and others 2000b, 2001) and no recent study on its effectiveness in Red Delicious apples. The extent of

browning varies greatly among apple cultivars (Coseteng and Lee 1987). Factors responsible for this were reported to be differences in PPO activity and its substrate content. Red Delicious apple was shown to have the highest PPO activity among many apple cultivars (Janovitz-Klapp and others 1989). The third objective of this study was, therefore, to investigate the antibrowning effectiveness of oxalic acid on fresh-cut surfaces of banana and Red Delicious apple. The fourth objective was to compare antibrowning effectiveness of oxalic acid to that of other structurally related carboxylic acids such as malonic, succinic, and glutaric acids using a machine-vision system. Machine vision, a nondestructive method, can rapidly quantify the disappearance of lighter colors and the formation of darker colors in nonuniformly colored surfaces of many foods (Luzuriaga 1999).

Ascorbic acid and its formulations are commonly used as an antibrowning agent in the manufacturing of fruit juices, purees, fresh and frozen sliced fruits, and canned fruits and vegetables. However, ascorbic acid alone is generally not a long-lasting antibrowning agent. Typically browning proceeds after the exhaustion of ascorbic acid; and the dehydroascorbic acid formed during the oxidation reaction is not involved in browning inhibition (Sapers and others 1989; McEvily and others 1992; Sapers 1993; Nicolas and others 1994). There is substantial interest in using ascorbic acid along with other inhibitors for better control of enzymatic browning. For example, a combination of ascorbic acid and citric acid was found to be more effective than ascorbic acid alone (Sapers and others 1989). This is probably due to the stability of ascorbic acid in an acidic environment and the acidic environment's effect on the catalytic activity of PPO. Ascorbic acid inhibits melanin formation by preventing the accumulation of *o*-quinones,

and/or may form stable colorless products (McEvily and others 1992; Sapers 1993; Nicolas and others 1994). Citric acid functions as a PPO inhibitor through its chelating action (McEvily and others 1992; Sapers 1993). Thus, the fifth objective of this study was to investigate the relative antibrowning potency of oxalic acid in conjunction with ascorbic acid to control browning of minimally processed apples and bananas.

In addition to its general occurrence in plants, PPO is also found in insects (Brunet 1980; Sugumaran 1998). During insect development, PPO is believed to be responsible for various physiological roles. It is involved in sclerotization (hardening) and tanning of the insect cuticle (Sugumaran 1998); and in defense reactions, wound healing, and disease resistance (Gillespie and others 1997; Sugumaran and Nellaiappan 2000). It stands to reason that natural inhibitors within the house fly regulate this development, and previous literature has indicated the presence of endogenous inhibitors to house fly PPO (Tsukamoto and others 1992; Daquinag and others 1995, 1999). There is little, if any, documentation on the potential applicability of these inhibitors to food systems. The sixth and final objective of this study was to provide evidence for the existence of apple PPO inhibitor(s) in the common house fly (*Musca domestica* L.) and to demonstrate inhibition of apple PPO activity using inhibitor(s) isolated from house fly pupae. The PPO inhibition profile of the house fly during its growth and development was also evaluated.

CHAPTER 2
LITERATURE REVIEW

Importance of Enzymatic Browning in the Food industry

Problem

Enzymatic browning is caused mainly by the action of polyphenol oxidase (PPO; 1,2-benzenediol:oxygen oxidoreductase; EC 1.10.3.1) (Nicolas and others 1994; Gooding and others 2001; Yoruk and Marshall 2003). PPO is a generic name for a group of enzymes capable of catalyzing reactions for several phenols to produce *o*-quinones (Mathew and Parpia 1971; Whitaker 1994). The catalytic action of PPO has a large impact on the quality of several fruit and vegetable crops and results in alteration of color, flavor, texture, and nutritional value (Vamos-Vigyazo 1981; Yoruk and Marshall 2003). It is a limiting factor in the handling and processing of crops because peeled, sliced, bruised, or diseased tissues rapidly undergo browning.

Some commercially important edible plant products susceptible to adverse browning reactions include fruits such as apple (Barrett and others 1991; Yemenicioglu and others 1997), avocado (Kahn and Pomerantz 1980), banana (Gooding and others 2001), cucumber (Miller and others 1990), grape (Rathjen and Robinson 1992), pineapple (Das and others 1997), mango (Robinson and others 1993), peach and apricot (Fraignier and others 1995); and vegetables such as eggplant (Perez-Gilabert and Carmona 2000), cabbage (Fujita and others 1995), lettuce (Heimdal and others 1994), potato (Cho and Ahn 1999), and table beet (Escribano and others 1997). However, browning in some other instances such as in the processing of black tea (Eskin 1990; Ullah 1991), coffee

(Amorim and Melo 1991), and cocoa (Lopez and Dimick 1991) is beneficial to some extent as it enhances the quality of the beverages by forming flavorful products.

In addition to its general occurrence in plants, PPO is also found in seafood (crustacean) products, such as shrimp (Simpson and others 1988) and lobster (Ferrer and others 1989). These highly prized and economically valuable products are vulnerable to deteriorative enzymatic browning, also referred to as melanosis. The millions of kilograms of seafood, specifically shrimp, caught or imported into processing facilities must be treated with chemical preservatives to control and/or eliminate this discoloration process (Kim and others 2000).

Biochemical Foundation of the Problem

The simplified scheme of PPO reactions and subsequent nonenzymatic condensation process involved in food deterioration is shown in Figure 2-1. Enzymatic browning occurs in two steps: The first step is the enzymatic oxidation of monophenols (monophenolase activity) or *o*-diphenols (diphenolase activity) to yield *o*-quinones. The second step is condensation or polymerization reactions (Whitaker 1995; Sanchez-Ferrer and others 1995; Yoruk and Marshall 2003).

The involvement of copper as a prosthetic group of PPO is essential for its activity (Solomon and Lowery 1993; Van Gelder and others 1997). In the absence of an *o*-diphenol (BH_2, acting as the electron donor), there is a characteristic lag or induction period in the monophenolase reaction prior to attaining a steady-state rate (Kahn and Pomerantz 1980; Sanchez-Ferrer and others 1993). When the existing active PPO generates some BH_2, the reaction continues as normal (Sanchez-Ferrer and others 1993; Whitaker 1994; Lerch 1995). Duration of the pronounced lag and the steady-state rate are experimentally influenced by a number of factors including temperature and pH; and

BH_2, oxygen, enzyme, and monophenol concentrations (Sanchez-Ferrer and others 1993; Espin and others 1997; Perez-Gilabert and Carmona 2000; Laveda and others 2001).

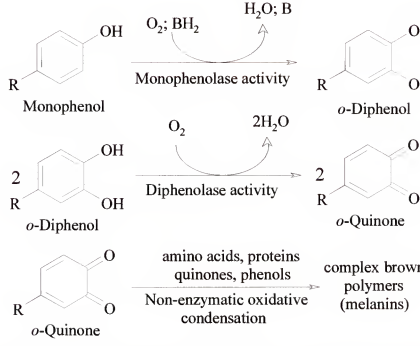


Figure 2-1. Enzymatic and subsequent nonenzymatic condensation processes for browning

The overall hydroxylation reaction involves introduction of one atom of oxygen onto the monophenol and reduction of the other to water (Mason 1957). Isotope studies with $^{18}O_2$/ $H_2{}^{16}O$ and $^{16}O_2$/ $H_2{}^{18}O$ showed that the origin of oxygen appearing in the monophenol is molecular oxygen (Mason 1956). In the case of diphenolase activity (Figure 2-1), BH_2 is not required because the existing *o*-diphenols are sufficient for the reaction (Whitaker 1995). Two molecules of *o*-diphenol are oxidized to two molecules of *o*-quinone. Since both atoms of the oxygen molecule are reduced to water, diphenolase activity is referred to as a four-electron transfer oxidase (Mason 1957).

Severe browning of plant products arises in stress conditions due to subcellular decompartmentation and oxygen penetration, leading to PPO-substrate contact. These

reactions are complex, given that a large number of monophenolic and/or diphenolic compounds catalyzed by PPO may in turn form a variety of products (quinones and condensation products) (Mathew and Parpia 1971; Vamos-Vigyazo 1981; Macheix and others 1990; Sugumaran 1998; Jimenez and Garcia-Carmona 1999; Yoruk and Marshall 2003). Typical reddish-brown color characteristic of enzymatic browning is due primarily to nonenzymatic secondary reactions of *o*-quinones resulting in formation of complex polymers known as melanins (Lerner 1953; Mathew and Parpia 1971; Vamos-Vigyazo 1981; Mayer and Harel 1991; Sapers 1993; Sanchez-Ferrer and others 1995; Whitaker 1995).

Oxidation products of phenolic compounds have the potential to interact with food proteins, leading to covalent condensations (Mason and Peterson 1965; Pierpoint 1966; Mathew and Parpia 1971; Nicolas and others 1994). Cross-linking reactions could result in changes in structural, functional and nutritional characteristics of food proteins (Matheis and Whitaker 1984). Reduction in the nutritive value of food proteins is due to the interactions of quinones with side chains of essential amino acids in plant proteins (Matheis and Whitaker 1984; Felton and others 1989, 1992a, 1992b). The –SH and $-NH_2$ groups of amino acids are mostly susceptible to binding or alkylation by quinones. A decrease in the available lysine content of casein in the presence of phenolic compounds and PPO was reported (Matheis and Whitaker 1984; Felton and others 1992a, 1992b). Felton and others (1989) also observed an 8% reduction in measurable lysine in tomato protein treated with chlorogenic acid and PPO.

Additionally, food proteins containing phenolic tyrosine groups or attached to a phenolic acid through a pseudopeptide bond are also subject to modification by serving

as substrates for PPO (Matheis and Whitaker 1984). For example, oxidation of tyrosine residues on alcohol dehydrogenase and aldolase by mushroom PPO was shown to result in about 50% and 80% loss in their catalytic activities, respectively (Cory and Frieden 1967). Subsequent studies by Ito and others (1984) identified the reaction products of PPO action on tyrosinase residues in proteins: 5-S-cysteinyl-3,4-dihydroxyphenylalanine (5-S-cysteinyl-dopa) was found to be the major product in yeast alcohol deydrogenase and bovine serum albumin, which contain cysteine residue(s); whereas dopa is formed in bovine insulin, which lacks cysteine residues. Moreover, quinones formed during PPO-oxidation reactions may undergo redox recycling, which generates free radicals, and can damage DNA, proteins, amino acids or lipids (Felton and others 1992a, 1992b; Hill 1992).

Polyphenol Oxidase (PPO)

A plethora of studies shows that PPO (also known as tyrosinase, polyphenolase, phenolase, catechol oxidase, cresolase, or catecholase) is widely found in nature (Whitaker 1994, 1995). PPO is typically found in plant tissues (Vamos-Vigyazo 1981; Zawistowski and others 1991; Sherman and others 1995; Fraignier and others 1995; Haruta and others 1999); in some fungi (Mayer and Harel 1979; Whitaker 1995); and in some animal tissues, including insects (Brunet 1980; Sugumaran 1998), humans (Osuga and others 1994), and crustaceans (Kim and others 2000). PPO is essentially associated with dark pigmentation of the tissues.

Molecular Mechanism of Action

The overall mechanism of monophenol and diphenol oxidations proposed for *Neurospora crassa* PPO is shown in Figure 2-2 (Lerch 1995). The mechanism of the catalytic action for the enzyme was deduced based on the geometric and electronic

structure of the copper active site (Himmelwright and others 1980; Lerch 1983, 1995; Wilcox and others 1985; Solomon and others 1992); which structurally resembles that found in hemocyanin, an oxygen transport protein (Himmelwright and others 1980; Gaykema and others 1984).

The active site of the enzyme consists of two copper atoms each in the ligand field with three conserved histidine residues (Lerch 1983; Huber and others 1985) and exists in three forms: met-PPO (Cu^{+2}), deoxy-PPO (Cu^{+1}), and oxy-PPO (Cu^{+2}). The resting form of the enzyme is believed to be met-PPO (Himmelwright and others 1980; Solomon and others 1992). It is reduced to deoxy-PPO via oxidation of one molecule of catechol to o-benzoquinone. The active site of the enzyme for the monophenol oxidation pathway (Figure 2-2) then combines with oxygen to give an oxy-PPO intermediate. Monophenolic substrates can only react with oxy-PPO, forming the ternary PPO-oxygen-monophenol complex (Lerch 1995). Formation of *o*-benzoquinone from monophenol is followed by its release from the enzyme. The PPO is converted into deoxy PPO and ready for another round without cycling through the resting form. In the diphenol oxidation pathway (Figure 2-2), diphenolic substrates react with oxy-PPO and also with met-PPO (Lerch 1995). Met-PPO is first reduced to its deoxy form with the oxidation of one molecule of *o*-diphenol and then combines with oxygen, leading to the formation of oxy-PPO, which in turn forms *o*-diphenol-PPO complex. After the oxidation of catechol to *o*-benzoquinone, the enzyme is reduced to its met form.

Even though it is not yet fully clear whether the mechanisms stated are applicable to all PPOs, it is evident that whatever the mechanism of the enzyme action, copper is essential for PPO activity. Removal of copper from the enzyme by copper-chelating

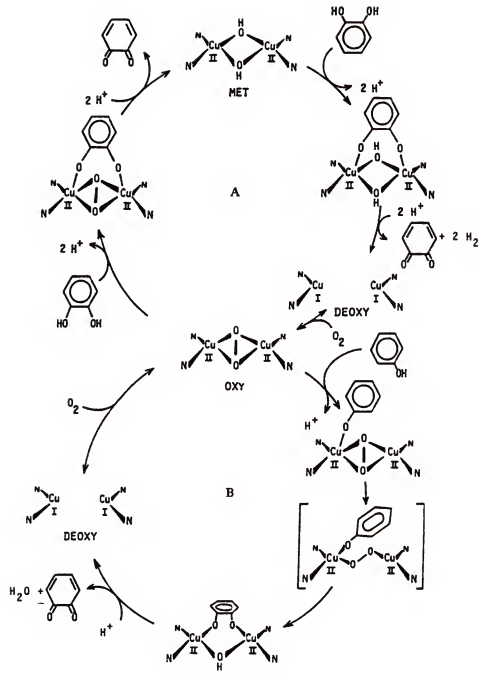


Figure 2-2. Proposed monophenol and diphenol oxidation pathways of *Neurospora crassa* PPO. A) Diphenol. B) Monophenol. Reproduced with permission from Lerch (1995), Enzymatic Browning and Its Prevention, ACS Symposium Series 600, p 76. Copyright 1995 American Chemical Society

reagents results in a relatively inactive enzyme whose activity can be restored by the addition of excess copper (Park and Luh 1985; Kowalski and others 1992). Based on amino acid sequences of PPOs from various plant, animal, fungal, and bacterial sources, all have 2 conserved copper-binding sites, called CuA and CuB (Steffens and others 1994; Lerch 1995; Van Gelder and others 1997; Haruta and others 1998; Chevalier and others 1999; Gooding and others 2001). This binuclear copper is the site of interaction between the enzyme and its substrates (Dietler and Lerch 1982; Wilcox and others 1985; Solomon and Lowery 1993; Steffens and others 1994).

In addition, sequence comparison studies have revealed high homology around the copper-binding ligands of arthropodan and molluscan hemocyanins and several PPOs (Steffens and others 1994; Boss and others 1995; Thygesen and others 1995; Van Gelder and others 1997; Haruta and others 1998; Chevalier and others 1999). Comparative studies on several plant PPOs show that they also have structural similarity in terms of immunological criteria (Flurkey 1986; Fraignier and others 1995; Haruta and others 1999). It is believed that the knowledge of hemocyanin whose structure is confirmed with X-ray analysis (Gaykema and others 1984) will be helpful in predicting a structural model for the active site of PPOs in the absence of X-ray crystallographic information (Van Gelder and others 1997).

Plant PPO

PPO is typically present in most plant tissues (Zawistowski and others 1991; Sherman and others 1995; Fraignier and others 1995; Haruta and others 1999). Because of its involvement in adverse browning of plant products, PPO has received much attention from researchers in the field of plant physiology and food science. The chemistry of PPO-catalyzed browning reactions in higher plants beyond the *o*-quinone

step (Figure 2-1) has been elucidated in only few cases, because of insolubility of the products (Whitaker 1995). The sequence of biochemical reactions leading to formation of melanin from the oxidation of phenolic amino acid tyrosine is shown in Figure 2-3. The products through indole-5,6-quinone are soluble in *in vitro* systems (Whitaker 1995).

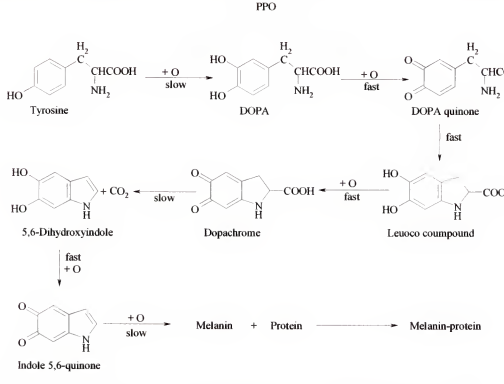


Figure 2-3. Enzymatic oxidation of the phenolic amino acid tyrosine to melanin. Reproduced with permission from Lerner (1953), Adv. Enzymol., 14, p 76. Copyright 1953 John Wiley and Sons, Inc

Even though oxidation of phenols and formation of melanins are normal physiological processes of PPO, the significance of the enzyme activity in living intact plant tissues is not fully understood. Since one must take into account the subcellular location of PPO to ascribe a physiological function to the enzyme (Mayer and Harel 1979), location of PPO has always been of special interest to researchers. Considerable evidence shows that plant PPO is a nuclear-coded protein (Lax and others 1984) and located in the plastids (Vaughn and Duke 1984; Vaughn and others 1988; Sherman and

others 1991; Murata and others 1997). It appears from the investigations that PPO is synthesized on cytoplasmic ribosomes and is practically inactive until integrated into the plastid (Yalovsky and others 1992; Marques and others 1995). Nuclear-coded precursor proteins of higher molecular weight, possessing a transit peptide sequence at the amino-terminal end, are directed to the chloroplast. Such signal sequences are eventually removed by specific stromal peptidases (Koussevitzky and others 1998) during the process of incorporation and a lower molecular weight mature protein is produced (Sommer and others 1994). This observation has been verified for a number of species, including apricot, apple, broad bean, grape, potato, spinach, tomato, and pokeweed (Yoruk and Marshall 2003).

Thylakoid location of the enzyme led researchers to suggest that PPO may play a role in photosynthesis of functional chloroplast (Vaughn and Duke 1984; Vaughn and others 1988). Association of the enzyme with photosystem complexes suggested a likely role in photosynthetic electron transport (Lax and Vaughn 1991; Sheptovitsky and Brudvig 1996). It may function by regulating pseudocyclic photophosphorylation (ATP production with oxygen as a terminal electron acceptor) (Vaughn and Duke 1984; Sherman and others 1995). However, pH-dependent activity of photosystem II membrane-associated PPO suggested that PPO is important mostly for dark processes in thylakoid lumen (Sheptovitsky and Brudvig 1996).

Involvement of PPO in oxidation of phenolic compounds in various plants is indisputably its most familiar role. Severe oxidative browning does not occur in healthy intact plant tissues, perhaps because PPO activity *in vivo* is limited by lack of phenolic substrates physically separated from the enzyme within the vacuoles (Vaughn and Duke

1984; Vaughn and others 1988; Macheix and others 1990; Amorim and Melo 1991; Lopez and Dimick 1991). It is usually believed that senescence or injury results in destruction of the biological barriers between PPO and polyphenols; and the enzyme is active only when it unites with its phenolic substrates (Vaughn and Duke 1984; Vaughn and others 1988; Felton and others 1989; Eskin 1990; Barrett and others 1991; Lopez and Dimick 1991; Murata and others 1997).

There is general agreement that during normal growth and development, PPO activity is much higher in unripe fruits and young leaves than in mature fruits and mature leaves (Lanker and others 1987; Felton and others 1989; Mayer and Harel 1991; Steffens and others 1994; Murata and others 1995; Serradell and others 2000), suggesting its possible role in protection of growing plants against infection or injury. The general decrease in PPO activity with tissue age may be attributable to conformational changes, degradation by proteases, a decline in concentration of latent enzyme activators or of phenolic substrate biosynthesis, (Barrett and others 1991; Murata and other 1995; Laveda and others 2000), or to inactivation of PPO due to tanning reactions (Lanker and others 1987; Macheix and others 1990). However, a large number of studies on plants proposed that the level of enzyme activity is controlled at the transcriptional level, as PPO transcripts are highly expressed at early developmental stages and expression decreases as development continues (Rathjen and Robinson 1992; Shahar and others 1992; Hunt and others 1993; Dry and Robinson 1994; Boss and others 1995; Thygesen and others 1995). Absence of translatable PPO mRNA at late ripening stages of apricot fruit in contrast to significant PPO activity suggests that translational and posttranslational controls may be involved in regulation and level of PPO activity (Chevalier and others

1999). Likewise, significant levels of PPO activity with little PPO gene expression throughout ripening of banana fruit suggests activation of pre-existing enzymes as a result of a loss in membrane integrity during natural ripening of the fruit (Gooding and others 2001).

Furthermore, elevation of PPO activities for numerous plants in response to pathogen and insect assaults is considered an additional line of the plants' defense against pathogens and pests (Mayer and Harel 1979; Bashan and others 1987; Felton and others 1989; Thipyapong and Steffens 1997). PPO activities could be induced locally at infected or wounded sites of the tissues (Boss and others 1995; Ray and Hammerschmidt 1998) or systemically perhaps to protect the plant against further attack (Thipyapong and others 1995) or both (Bashan and others 1987; Constabel and others 1995; Thipyapong and Steffens 1997). Hence, either constitutive, induced or both types of PPO activity may be involved in the putative protective defense strategy. Thipyapong and Steffens (1997) indeed reported that anti-sense down regulation of constitutive and induced PPO activity results from increased pathogen susceptibility. For the interested reader, many of these facts including physicochemical properties and potential physiological roles of PPO in plants are detailed by Yoruk and Marshall (2003).

Whatever the mechanism is to control the level of PPO activity, disruption of membrane integrity, loss of latency, and transcriptional or posttranscriptional control during normal growth and development (as reviewed by Yoruk and Marshall 2003), PPO is present at all developmental stages of plants and could even be more abundant after wounding or infection. Numerous studies put forward the role of PPO in protection of plants against diseases and invading pathogens or insect pests (Boss and others 1995;

Constabel and others 1995; Thipyapong and others 1995; Thipyapong and Steffens 1997; Ray and Hammerschmidt 1998). This implication is essentially based on the remarks that secondary reaction products of PPO prevent spread of infection. The most extensively accepted potential effects of the reaction products on disease resistance could be classified into 3 groups (Yoruk and Marshall 2003):

- Oxidative polymerization of quinones form insoluble melanin over wounds, sealing off infected tissues.
- Bacteriocidal and fungicidal effect of hydroxyphenolics and quinones to assaulting microorganisms and toxic effects of the polymerized phenolics on invading viruses in injured tissues.
- Covalent modification of proteins by quinones as an antinutritive defense mechanism. The quinones generated by reaction of PPO with a variety of phenolic substrates can modify dietary proteins by reacting with amino, sulfhydryl, phenolic and imidazole groups, reducing their nutritive value to insect herbivores.

General Properties of Apple and Banana PPO

Apples and bananas are among the most popular global fruit crops in the world (Nicolas and others 1994; Becker and others 2000). World production of apples and bananas was estimated to exceed 35 and 85 million tons/yr, respectively (Nicolas and others 1994; Becker and others 2000). Bananas are one of the main crops in tropical and subtropical developing countries (Becker and others 2000). Given that the ripe fruits are extremely prone to adverse enzymatic browning during handling and processing, the fruit industry faces the problem of how to prevent browning of minimally processed crops. Browning has a large impact on the quality of bananas and apples and their products as it results in alteration of color, flavor, texture, and nutritional value (Sapers 1993; Nicolas and others 1994; Gooding and others 2001). It is, therefore, necessary to maintain the original color and thereby quality of the fresh raw material during food processing and in the market. Biochemical characteristics of PPOs from apples and bananas have received

particular attention from researchers all over the world because an understanding of the essential factors controlling the action of the enzyme is necessary in an attempt to inhibit or control its activity in these commodities (Nicolas and others 1994; Gooding and others 2001; Yoruk and Marshall 2003).

Substrate specificity

Several phenolic compounds serve as substrates for PPOs from apple and banana (Table 2-1). Catechol and 4-methylcatechol, frequently used to measure PPO activity, are not natural substrates since they do not accumulate in fruits (Macheix and others 1990). Types and relative concentrations of natural phenols vary widely for different plant sources. Catechin, epicatechin and caffeic acid derivatives are common natural substrates of fruit PPOs (Macheix and others 1990). Chlorogenic acid and catechin are the major phenolic compounds in mature and immature apples, respectively (Murata and others. 1995). Yang and others (2001) reported that browning of banana is mainly due to the enzymatic oxidation of dopamine.

Distinct variations in the activities of apple and banana PPOs for different types of substrates are shown in Table 2-1. PPO is active to those substrates with a high preference to the enzyme. Nature of the side chain, numbers of hydroxyl groups and their position in the benzene ring of the substrate have a major effect on the catalytic activity of the enzyme (Montgomery and Sgarbieri 1975; Park and Luh 1985; Macheix and others 1990). In the case of apple PPO, 4-methylcatechol is oxidized at much faster rates than other structurally related substances (Table 2-1). The substrates catechol and chlorogenic acid also show relatively high levels of activity. The activity of apple PPO on tyrosine is much lower than on *o*-diphenols (Zhou and others 1993), which is typical for plant PPOs (Yoruk and Marshall 2003). However, the activity of PPO from banana is greatest with

dopamine followed by catechol and *d*-catechin (Table 2-1). Previous studies on banana PPO by Montgomery and Sgarbieri (1975) also showed that the enzyme is more specific for *o*-diphenols, dopamine, catechol and 4-methylcatechol, and tyrosine was oxidized at a very slow rate.

Table 2-1. Relative substrate specificity of PPOs from apple and banana

	Percent activity		
Substrates	Apple [a]	Banana [b]	References
4-Methylcatechol	181		[a] Zhou and others (1993)
Catechol	100	34	[b] Yang and others (2001)
Chlorogenic acid	102	5	
d-Catechin	54	12	
Epicatechin		9	
Caffeic acid		1	
Pyrogallol	38	1	
Dopamine	37	100	
DL-DOPA	12	8	
Tyrosine	3		

DOPA: Dihydroxyphenylalanine

Substrate specificity of PPOs also differs among varieties. For instance, Red Delicious (Stelzig and others 1972) and Monroe apple PPOs (Zhou and others 1993) show highest activity toward 4-methylcatechol whereas Amasya apple PPO oxidizes catechol more rapidly than 4-methylcatechol (Oktay and others 1995). Experimental factors such as extraction and assay methods used during determination also affect the substrate specificity of PPOs (Stelzig and others 1972; Nicolas and others 1994). In addition, PPO isoforms in a tissue of interest may also exhibit different substrate specificities and variations in their relative activities toward phenolic compounds (Harel and others 1965; Park and Luh 1985; Thomas and Janave 1986; Oba and others 1992). Two isoforms isolated by Oba and others (1992) in banana bud extracts showed maximum relative activity toward dopamine. However, these enzymes exhibited variations in their activities toward chlorogenic acid, L-DOPA, and (+)-catechin. Four

PPO isoforms differing in substrate specificities also were found in apple tissues (Harel and others 1965).

Effect of pH and temperature

In general, pH is an important factor that significantly influences the catalytic activity of PPO. Changes in ionization of prototropic groups in the active site of an enzyme at lower acid and higher alkali pH values may prevent proper conformation of the active site, binding of substrates, and/or catalysis of the reaction (Tipton and Dixon 1983; Whitaker 1994). In addition, irreversible denaturation of the protein and/or reduction in the stability of the substrate as a function of pH could also influence the catalytic activity of enzymes. Kinetic behavior of PPO was reported to alter depending on the pH of the assay due to pH-induced conformational changes in the enzyme (Janovitz-Klapp and others 1989; Valero and Garcia-Carmona 1992, 1998).

The pH optimum of PPO varies widely with plant source but is generally in the range of 4.0-8.0 (Heimdal and others1994; Fraignier and others 1995; Marques and others 1995; Sheptovitsky and Brudvig 1996; Das and others 1997; Perez-Gilabert and Carmona 2000). The acidic pH optimum is common for apple PPO. However, some researchers found a single pH optimum at pH 4.5 to 5.0 (Janovitz-Klapp and others 1989; Zhou and others 1993), whereas others noted the existence of two pH optima, one at around pH 7.0 and one at around pH 5.0 (Stelzig and others 1972). Experimental factors such as extraction methods, temperature, and buffer system used during determination; and source differences can affect the optimum pH value for PPO (Stelzig and others 1972; Janovitz-Klapp and others 1989; Zhou and others 1993; Whitaker 1994). Nature of the substrate used in the activity assay is another factor, which may significantly influence the pH optimum of the enzyme. Apparent pH optima for Monroe apple PPO

were 4.6 with 4-methylcatechol and 5.0 with catechol (Zhou and others 1993). However, PPO from Amasya apple showed maximum activity at pH 7.0 with catechol but a pH of 6.6 with L-DOPA (Oktay and others 1995). This variation could be accounted for by differences in the binding ability of the substrates to the active site under acidic and alkaline conditions (Tipton and Dixon 1983; Whitaker 1994). The optimum pH of banana PPO for dopamine oxidation was reported to be 6.5 (Yang and others 2001). However, Oba and others (1992) reported 2 PPO isoforms in banana bud extracts with optimum pHs of 6.8 and 5.5 for the oxidation of dopamine. PPO isoforms present in apple tissue was also shown to exhibit different pH optima (Harel and others 1965; Marques and others 1995).

The pH for maximum PPO activity is also subject to changes when assayed in the presence of the modulator, sodium dodecyl sulfate (SDS). The general behavior of apple PPO with changing pH was altered in the presence of SDS (Marques and others 1995). Regardless of the substrate, the activity of apple PPO is inhibited at acidic pH and activated at pH above 5.0 in the presence of 3.5 mM SDS (Marques and others 1995). The pH optimum of the enzyme is also shifted from low to a higher pH value with different values for different substrates. This also appears to be the case for Prunus fruits (Fraignier and others 1995) and broad bean (Moore and Flurkey 1990; Jimenez and Garcia-Carmona 1996) PPOs, where SDS causes a shift in the pH optimum of the enzyme from low to higher pH values. In the case of PPOs from apple and Prunus fruits, modification of the behavior of the enzyme with regard to pH by SDS is not considered to be by activation of latent PPO form (Fraignier and others 1995; Marques and others 1995). In contrast, broad bean PPO is believed to exist in a latent state and is activated

several fold by SDS (Moore and Flurkey 1990). The difference in pH profiles of the latent and SDS-activated PPO forms was attributed to the displacement of the sensitive pKs of the enzyme caused by the interaction with negatively charged SDS molecules (Jimenez and Garcia-Carmona 1996; Espin and Wichers 1999).

Temperature is another important factor that significantly influences the catalytic activity of PPO. It is well known that a decrease in the internal energy of the reactant molecules at low temperatures corresponds to a slower reaction (Laidler and Peterman 1983; Lehninger and others 1993). In addition, integrity of the delicate three-dimensional structure of the enzyme molecule is subjected to disruption and denaturation at high temperatures (Whitaker 1994). Variations in temperature may also alter the solubility of oxygen, one of the substrates required for PPO (Figure 2-1) to perform its catalytic activity (Whitaker 1994).

The optimum temperature of PPO activity also varies for different plant sources. PPOs from apple (Zhou and others 1993) and banana (Yang and others 2001) have temperature optima of 30 °C under the specified experimental conditions. Banana PPO was relatively stable at high temperatures: about 90% of the activity remained after heat treatment at 60 °C for 30 min. Thermal inactivation of PPOs from several sources was shown to follow first-order kinetics (Lee and others 1983; Wesche-Ebeling and Montgomery 1990; Robinson and others 1993; Yemenicioglu and others 1997). A complete study on heat inactivation kinetics for a number of apple cultivars at 68, 73, and 78C showed that PPO activity initially increased, attributed to the activation of a latent form, and then decreased with heat, following a first order kinetic model (Yemenicioglu and others 1997). They also highlighted the variability in the thermal stability of the

apple cultivars; PPO in Amasya, for instance, is less heat-stable than PPO in Starking Delicious apples.

Insect PPO

In addition to its general occurrence in plants, PPO is also found in insects (Brunet 1980; Hara and others 1993; Sugumaran 1998). There is much similarity in molecular biology of PPOs from plants and insects. Studies on molecular cloning of PPOs from different insect species have also revealed 2 conserved copper-binding sites showing high sequence similarity to arthropod hemocyanins (Sugumaran 1998). However, insect PPOs differ from plant PPOs in that they lack signal peptide sequences, directing nuclear-coded precursor of plant PPOs to the chloroplast envelope for subsequent processing, as mentioned above. The enzyme appears to be located in the cuticle and hemolymph of insect blood (Brunet 1980).

In insects, PPO in blood, catalyzing tyrosine derivatives, exhibited both monophenolase and diphenolase activity, whereas cuticle PPO seems to show the latter activity exclusively (Figure 2-1) (Brunet 1980). Diphenolase activity is the most prevalent function of the enzyme, as all known PPOs are capable of oxidizing *o*-diphenols, irrespective of whether the sources are plants or insects. PPO activity during metamorphosis of insects drastically fluctuates (Yamaura and others 1980; Hara and others 1991, 1993). House fly goes through a complete metamorphosis including egg, larva, pupa, and adult stages (West 1951). The highest PPO activity was observed in the final instar larvae crawling from food to pupariate (Yamaura and others 1980; Hara and others 1991, 1993). It appears that the enzyme is useful to the larva during puparation in which the larval cuticle of the final instar is hardened into puparium.

The general properties of PPO action appear to be similar for both plant and animal PPOs; the enzyme acts upon a number of phenolic substrates and oxidizes these into quinone compounds (Figure 2-1) (Sugumaran 1998; Yoruk and Marshall 2003). Melanin biosynthesized in animals mainly by PPO acts as a skin and hair pigment-protecting agent against harmful solar radiation (Hill 1992). Additionally, PPO is responsible for various physiological roles in the development of insects, including cuticular hardening, wound healing, and defense reactions (Hopkins and Kramer 1992; Andersen and others 1996; Gillespie and others 1997; Sugumaran 1998).

The insect integument is composed of the cuticle and the underlying epidermal cells that secrete the cuticle (Richards 1978; Andersen 1979; Hepburm 1985). The biochemical components of cuticles include the polysaccharide chitin, structural proteins, enzymes, phenols and quinones, lipids, and inorganic compounds (Richards 1978; Andersen 1979; Hepburm 1985; Sugumaran 1998). The properties of cuticles vary both spatially and temporally according to the demands of function and growth due perhaps to the need for differential strengthening of the cuticle (Andersen 1979; Hepburm 1985; Andersen and others 1996; Sugumaran 1998). However, not all factors leading to these differences are known (Sugumaran 1998). The cuticle forms the exoskeleton of insects, supports muscle attachment, serves as permeability barriers by minimizing water loss, and plays a role in protection by preventing the entry of pathogens and insecticides (Richards 1978; Hepburm 1985; Andersen and others 1996). Characteristics of the cuticle also determine body shape, size, and coloration (Richards 1978; Hepburm 1985).

The major roles of PPO in formation of the exoskeleton of insects are related to sclerotization (hardening) and tanning of the insect cuticle (Hopkins and Kramer 1992;

Andersen and others 1996; Sugumaran 1998). Cuticular sclerotization is the process of cross-linking protein-to-protein chains, chitin-to-chitin chains, and possibly protein to chitin chains (Hopkins and Kramer 1992; Sugumaran 1998). The cross-linking or sclerotizing agents are quinones, quinone methides and semiquiones (Hopkins and Kramer 1992; Andersen and others 1996; Sugumaran 1998). PPO oxidizes phenolic compounds to the corresponding quinones, which react with certain side groups on adjacent protein chains and chitin forming adducts, thus linking proteins to each other and possibly to chitin (Hopkins and Kramer 1992; Sugumaran 1998). Quinones can react with thiol, amine, imidazole, hydroxyl and carboxyl groups (Hopkins and Kramer 1992; Sugumaran 1998). Sclerotization gives strength and rigidity to the cuticle (Hopkins and Kramer 1992). The greater the degree of sclerotization, the harder the cuticle. The chitin content does not control hardness of the cuticle, but sclerotization does (Andersen and others 1996; Sugumaran 1998).

Additionally, PPO in insects has been postulated to play critical roles in defense reactions, wound healing, and disease resistance (Gillespie and others 1997; Sugumaran and Nellaiappan 2000). Infection in insects stimulates a multifaceted defensive response (Gillespie and others 1997). These responses often result in activation of the PPO zymogen present in the hemolymph. It appears that potential effects of secondary reaction products of PPOs fulfill certain functional roles in both plants (previously mentioned) and insects (Gillespie and others 1997; Sugumaran and Nellaiappan 2000; Yoruk and Marshall 2003). In the case of insects, oxidative polymerization of quinones forms insoluble melanin over wounds and prevents loss of blood by sealing off the infected tissues (Gillespie and others 1997; Sugumaran and Nellaiappan 2000).

Melanization is also important in defense reactions of insects through its action in pathogen sequestration (Gillespie and others 1997). A melanin coat encapsulates the foreign invading organisms when they are too large to be phagocytosed. Bacteriocidal effect of quinones is considered an additional defense mechanism to assaulting microorganisms (Pye 1974).

Regulation of PPO activity during growth and development is vital for all animals. PPO in the open circulatory system of insects occurs as a proenzyme (inactive form), which is then activated at the required time (Hara and others 1993; Sugumaran and Nellaiappan 2000). The regulatory biochemical mechanisms of endogenous PPO activity include inactivation, polymerization, complexation with other proteins, and inhibition (Sugumaran and Nellaiappan 2000). It is believed that PPO activity could also be regulated by intrinsic inhibitors in the course of development (Tsukamoto and others 1992; Daquinag and others 1995, 1999; Sugumaran and Nellaiappan 2000). Tsukamoto and others (1992) isolated inhibitors from the pupal extracts of house fly and demonstrated inhibition on house fly PPO. The potent house fly PPO inhibitors were found to be heat-stable low-molecular-weight (approximately 3000 to 4200) peptides with an inhibition constant in the nM range (Tsukamoto and others 1992; Daquinag and others 1995). Additionally, Sugumaran and Nellaiappan (2000) have isolated a high molecular weight (380000) glycoprotein inhibitor of PPO from the larval cuticle of the tobacco horn worm, *Manduca sexta*, and proposed that the presence of endogenous PPO inhibitor(s) would ensure the direct control of PPO activity in insects. The specific endogenous PPO inhibitor of *Manduca sexta* inhibited the activity of not only insect PPOs, but also plant PPOs and laccase.

Inhibition/Prevention of Enzymatic Browning

Millions of dollars in crop losses occur yearly due to enzymatic browning (Martinez and Whitaker 1995; Kim and others 2000). Significant economic impact of PPO-induced deleterious browning reactions in fruit and vegetables, as well as seafood, such as shrimp and lobster, necessitates its control to maintain quality and to extend product shelf-life. Several approaches can be shown experimentally to diminish or prevent browning of injured tissues. As reviewed by McEvily and others (1992), inhibitors available to suppress PPO activity fall into 6 groups based on their mode of action:

- Reducing agents (ascorbic acid and analogues, sulfites)
- Chelating agents (ethylenediaminetetraacetate (EDTA), sodium diethyldithiocarbamate (DIECA), sodium azide)
- Complexing agents (cyclodextrins, chitosan)
- Acidulants (ascorbic acid, citric acid, malic acid, phosphoric acid)
- Enzyme inhibitors (substrate analogs, halides)
- Enzyme treatments (proteases, *o*-methyltransferase)

These compounds diminish or inhibit the browning reaction rate by means of eliminating from the reaction an active reaction element(s), that is, enzyme, substrate(s), copper (prosthetic group) or a reaction intermediate (*o*-quinones) (Vamos-Vigyazo 1981; Macheix and others 1990; Nicolas and others 1994; Ahvenainen 1996; Ferrar and Walker 1996). PPO from different sources may react similarly with inhibitor compounds. However, effectiveness of inhibitors against different PPOs could vary significantly and, therefore, specific control measures for individual systems would also be needed (Ferrar and Walker 1996).

Reducing agents are broadly used in the food industry. They inhibit melanin formation by preventing the accumulation of *o*-quinones, or they may form stable colorless products (Nicolas and others 1994; Osuga and others 1994; Ashie and others 1996; Kim and others 2000). Of those agents, one of the most potent inhibitors of enzymatic browning is sulfur dioxide (SO_2) or sulfites (sodium sulfite and sodium bisulfite) (Sapers 1993; Kim and others 2000). Sulfiting agents were utilized broadly in the fruit and vegetable industry as antibrowning agents because of their effectiveness and low cost (Eskin 1990; Sapers 1993). It is believed that sulfites not only simply act as a reducing agent but also have ability to directly inhibit PPO. They also interact with quinones preventing their further participation in forming brown pigments (Ashie and others 1996). However, due to safety concerns, their use in fresh fruit and vegetables was banned by the Food and Drug Administration (Sapers 1993; Martinez and Whitaker 1995). They are still allowed for use on shrimp to delay the formation of blackspot and maintain the quality during storage or processing (Kim and others 2000).

Ascorbic acid (vitamin C), the best-known alternative reducing agent to sulfite, and its formulations are commonly used as an antibrowning agent in the manufacturing of fruit juices, purees, fresh and frozen sliced fruits, and canned fruits and vegetables. Ascorbic acid retards the formation of melanins by essentially reducing *o*-quinones back to the corresponding reactants, *o*-diphenols, before the quinones can undergo additional oxidative polymerization as shown in Figure 2-4. However, ascorbic acid is generally not a long-lasting antibrowning agent (Sapers 1993). Typically browning proceeds after the exhaustion of ascorbic acid because the dehydroascorbic acid accumulating during the

oxidation reaction is not a PPO inhibitor (McEvily and others 1992; Sapers 1993; Osuga and others 1994; Ashie and others 1996).

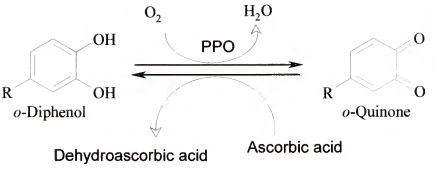


Figure 2-4. Mechanism of prevention of enzymatic browning by ascorbic acid

There is substantial interest in using ascorbic acid along with other inhibitors for better control of PPO-induced browning. For example, a combination of ascorbic acid and citric acid was found to be more effective than ascorbic acid alone (Eskin and others 1971; Sapers 1993). This is probably due to the stability of ascorbic acid in an acidic environment as well as inhibition of the acidic environment on the catalytic activity of the enzyme (Yoruk and Marshall 2003). Citric acid may also function as a PPO inhibitor through its chelating action (Eskin and others 1971); and ascorbic acid through the site-directed specificity toward histidine residues on the PPO protein (Golan-Goldhirsh and others 1992; Osuga and others 1994).

Chelating agents would act as a PPO inhibitor by making the active site copper unavailable since they have the ability of reacting with metals through chelation (McEvily and others 1992). Copper is crucial for any PPO activity for the reason that the enzyme is a copper metalloprotein (Lerch 1995; Van Gelder and others 1997). The active site of the enzyme comprises two copper atoms through which the enzyme interacts with its phenolic substrates and oxygen (Lerch 1995; Van Gelder and others 1997). Some agents serving as chelators for copper include EDTA, phosphates, citric acid, DIECA,

and sodium azide (Eskin and others 1971; McEvily and others 1992). In a chelator-metal complex, the chelator functions as a Lewis base (electron-donating species) and the metal ion acts as a Lewis acid (electron-accepting species) (Miller 1996).

The use of acidulants is another approach widely used in food processing to diminish browning. Acids naturally present in some edible food products, such as ascorbic, citric, malic, or phosphoric, shift the food pH to 3 or lower (Park and Luh 1985; Eskin 1990; Osuga and others 1994). Lemon juice is widely added to foods in substantial quantities to prevent enzymatic browning. PPO is generally catalytically more active in a pH range from 4.0-8.0 and the enzyme activity drastically drops at a higher acid environment (Yoruk and Marshall 2003). Mode(s) of pH-induced inhibition is attributable to protonation of catalytic groups essential for catalysis, conformational changes in the active site of the enzyme, irreversible denaturation of the protein, and/or reduction in the stability of the substrate as a function of pH (Tipton and Dixon 1983; Whitaker 1994). Additionally, acidity may also diminish the strong binding of the enzyme to its active site copper, increasing ability of citric acid to react with copper through chelation (Osuga and others 1994).

An alternative way of controlling adverse browning is the use of physical treatments. These include heating, freezing, refrigeration, dehydration, irradiation and high pressure (Ashie and others 1996; Kim and others 2000). However, these methods may also have some disadvantages, such as subcellular decompartmentation leading to enzyme-substrate contact and deterioration in texture (Macheix and others 1990). Packaging under controlled atmospheres and the use of edible films and coatings as part of the food product, which prevents the penetration of oxygen, are other appealing

methods for extending postharvest storage life of processed fruit and vegetables (Ahvenainen 1996).

Only a few browning inhibitors have the potential for use in the food industry because of off-flavors/odors, food safety, economic feasibility and perhaps effectiveness of inhibition as well (Eskin 1990; McEvily and others 1992; Sapers 1993; Park 1999; Chen and others 2000). Therefore, there is interest to find additional effective, natural and safe antibrowning agent(s) for preventing adverse browning reactions in fruit and vegetables. Substantial investigations are performed on natural antibrowning substances, including amino acids (Kahn 1985), Maillard reaction products synthesized from various amino acids with glucose (Tan and Harris 1995), honey (Oszmianski and Lee 1990; Chen and others 2000), and pineapple juice (Lozano-de-Gonzalez and others 1993). These natural inhibitors of PPO are not yet commercially utilized for food practice.

The more recent approach to reduce browning capacity of plants and, in turn, to improve crop quality is the control of PPO levels *in vivo* by means of antisense RNA strategies (Steffens and others 1994; Martinez and Whitaker 1995). The idea behind this approach is that the mRNA encoded by the antisense gene hybridizes with mRNA encoded by endogenous gene and consequently no translation occurs (Martinez and Whitaker 1995). Significant advances made on the molecular biology and genetics of PPO are discussed by Steffens and others (1994), Van Gelder and others (1997) and Yoruk and Marshall (2003). In light of the molecular and genetic data available, it is believed that manipulation or regulation of the PPO gene expression to diminish adverse effects of PPO on quality of fruit and vegetable crops without application of food additives will be the future challenge. Anti-sense down regulation of PPO, for instance, in

potato has already been performed by Bachem and others (1994) and Coetzer and others (2001). Genetic transformation has also been performed by Murata and others (2000) who showed that a transgenic apple (*Malus* x *domestica*) shoot in which PPO expression is reduced has lower browning potential than a control shoot.

Oxalic Acid

There are few reports on the inhibition of PPO by oxalic acid. Studies by Sato (1980a) demonstrated oxalic acid as a naturally occurring inhibitor of PPO in spinach. Ferrar and Walker (1993) reported that oxalic acid produced by the phytopathogen *Sclerotinia sclerotiorum* inhibited PPO activity in infected apples and bean pods. Oxalic acid inhibited the activities of PPOs from sunflower (Marciano and others 1983), sweet cherry (Pifferi and others 1974), and mushroom (Yong and others 1990; Son and others 2000a). Pifferi and others (1974) reported that oxalic acid showed a lower inhibitory effect on PPO from sweet cherry fruit than malonic acid, but higher than succinic acid. Glutaric acid was not an efficient inhibitor at all. However, oxalic acid was shown to be as effective an inhibitor as kojic acid and more potent than cysteine and glutathione on the catechol-mushroom PPO reaction (Son and others 2000a).

Oxalic acid in both soluble and insoluble forms is a natural component of many higher plants; and it was suggested to have some selective, functional significance or adaptation during normal plant development (Ilarslan and others 1997). Although it is not an approved food additive, oxalic acid is a common component of a large number of foods from plant origin such as asparagus, broccoli, Brussels sprouts, carrot, garlic, lettuce, onion, parsley, pea, potato, purslane, spinach, tomato, turnip etc. (Table 2-2, USDA 1984). It was also found in cocoa, rhubarb, and several wild edible plants (Guil and others 1996; Son and others 2000b). The daily consumption of oxalic acid in Western

diets was reported to be within the range of 70 to 150 mg (Son and others 2001). Oxalic acid at high concentrations has a tendency to reduce calcium and magnesium bioavailability in humans (Prenen and others 1984; USDA 1984). It was, however, reported that these oxalic acid rich foods usually containing sufficient calcium or magnesium would not interfere with the calcium and magnesium availability of other foods in the diet (USDA 1984).

Table 2-2. Estimated oxalic acid content of some common foods of plant origin

Plant	Oxalic acid (mg/100g)	Plant	Oxalic acid (mg/100g)
Asparagus	130	Garlic	360
Beans, snap	360	Lettuce	330
Broccoli	190	Parsley	1700
Brussels sprouts	360	Pea	50
Cabbage	100	Purslane	1310
Carrot	500	Radish	480
Cauliflower	150	Spinach	970
Celery	190	Sweet potato	240
Cucumbers	20	Tomato	50
Eggplant	190	Turnip	210

Reference, USDA (1984)

CHAPTER 3
MODE OF INHIBITION OF POLYPHENOL OXIDASE BY OXALIC ACID

Introduction

Polyphenol oxidase (PPO) is a generic name for a group of enzymes capable of catalyzing reactions for several phenols to produce *o*-quinones (Mathew and Parpia 1971; Whitaker 1994). Based on amino acid sequences of PPOs from various plant, animal, fungal, and bacterial sources, all have 2 conserved copper-binding sites (Van Gelder and others 1997). The mechanism of the catalytic action for PPO was deduced from the geometric and electronic structure of the copper-active site (Lerch 1995), which structurally resembles that found in hemocyanin, an oxygen transport protein (Gaykema and others 1984). This binuclear copper is the site of interaction between the enzyme and its substrates (Lerch 1995).

PPO-catalyzed browning is of significant importance in the fruit and vegetable industry. It is a limiting factor in the handling and processing of crops as peeled, sliced, bruised, or diseased tissues rapidly undergo browning and this results in alteration of color, flavor, texture, and nutritional value (Mathew and Parpia 1971; Matheis and Whitaker 1984; Yoruk and Marshall 2003). Significant economic impact from enzymatic browning in the food industry necessitates its control in order to maintain quality and extend product shelf-life (Martinez and Whitaker 1995; Yoruk and Marshall 2003). Several approaches can be shown experimentally to diminish or prevent browning of injured tissues (McEvily and others 1992; Son and others 2001). However, only a few browning inhibitors have the potential for use in the food industry because of

off–flavors/odors, food safety, economic feasibility, and effectiveness of inhibition (Eskin 1990; McEvily and others 1992; Park 1999; Chen and others 2000). Although PPOs from diverse plant sources have similar structural and functional characteristics (Van Gelder and others 1997; Yoruk and Marshall 2003), the performance of compounds as browning inhibitors on PPOs from different plant sources could vary significantly and, therefore, specific control measures for individual systems would also be needed (McEvily and others 1992; Ferrar and Walker 1996).

Sulfiting agents were utilized broadly in the fruit and vegetable industry as antibrowning agents because of their effectiveness and low cost (Eskin 1990). However, due to safety concerns, their use in fresh fruit and vegetables was banned by the Food and Drug Administration (Sapers 1993). Ascorbic acid, the best-known alternative reducing agent to sulfite, and its formulations are commonly used as an antibrowning agent in the manufacturing of fruit juices, purees, frozen sliced fruits, and canned fruits and vegetables. However, browning proceeds after the exhaustion of ascorbic acid because the dehydroascorbic acid accumulating during the oxidation reaction does not participate in the reduction process (McEvily and others 1992; Whitaker 1994). Hence, there is interest in alternative antibrowning agents for better control of enzymatic browning in fruits and vegetables.

Oxalic acid at controlled concentrations has been proposed as an alternative antibrowning agent for certain practical applications (Son and others 2000a, 2000b, 2001). Although it is not an approved food additive, oxalic acid is a common component of a large number of foods from plant origin such as spinach, rhubarb, parsley, carrot, purslane, potato, cocoa, and several wild edible plants (Sato 1980a; USDA 1984; Guil

and others 1996; Son and others 2000b). Compared to a number of antibrowning agents, oxalic acid, similar to cysteine and kojic acid, effectively suppressed color deterioration in Liberty apple slices at concentration of 0.05% (Son and others 2001). There are few reports on the inhibition of PPO by oxalic acid. Studies by Sato (1980a) demonstrated oxalic acid as a naturally occurring inhibitor of PPO in spinach. Ferrar and Walker (1993) reported that oxalic acid produced by the phytopathogen *Sclerotinia sclerotiorum* inhibited PPO activity in infected apples and bean pods. Oxalic acid inhibited the activities of PPOs from sunflower (Marciano and others 1983), sweet cherry (Pifferi and others 1974), and mushroom (Yong and others 1990; Son and others 2000a). There is limited information on the inhibition mechanism of oxalic acid on PPO. The objectives of this chapter were to define the mode of oxalic acid inhibition on PPO, and to compare its inhibitory effectiveness to some other structurally related compounds including malonic, glutaric, and succinic acids.

Materials and Methods

Preparation of PPO

Apple PPO was extracted from apple acetone powder. Acetone powder was prepared from Red Delicious apples obtained from a local grocery store (Yemenicioglu and others 1997). Small pieces of peeled apple pulp (200 g) were homogenized in a prechilled Waring blender with acetone (-20 °C, 400 mL) for 1 min, and then filtered through a Buchner funnel using Whatman No 1 filter paper. The residue was reextracted three times with 200 mL of cold acetone. The resulting white powder was vacuum-dried at room temperature (22 °C) and stored in commercial vacuum bags at –20 °C until used for PPO extraction.

The modified procedures of Murata and others (1995) and Yemenicioglu and others (1997) were used for PPO extraction. The acetone powder (1 g) was incubated in 50 mL of 0.1 M KH_2PO_4/Na_2HPO_4, pH 7.2 buffer, containing 1% Triton X-100 (Bio-Rad Laboratories, Hercules, CA) for 20 min while stirring with a magnetic stirrer at 4 °C. The suspension was centrifuged for 30 min at 12000 x g, and the resulting supernatant filtered through glass wool was tested for PPO activity. PPO activity of apple acetone extract kept in the freezer (-20 °C) remained stable for a week with no visible intrinsic phenolic oxidation.

Preparation of Oxalic Acid Solutions

Oxalic acid stock solutions were prepared using oxalic acid dihydrate (Sigma Chemical Co., St. Louis, MO). In order to maintain the pH of the reaction system constant at any oxalic acid concentration, the pH of the stock oxalic acid solutions (stock A), 8, 16, 24, 32, 64, 96, 128, and 160 mM, prepared in 0.2 M sodium acetate-acetic acid, pH 5.0 buffer (25 mL) was adjusted to pH 5.0 with NaOH. The final volume of each stock solution was brought to 40 mL with the pH 5.0 buffer to obtain the final oxalic acid concentrations (final stock) of 5, 10, 15, 20, 40, 60, 80, and 100 mM. Final stock solutions were used in the assay to study PPO inhibition.

In experiments designed to study the effect of pH and oxalic acid concentration on the inhibition of PPO, oxalic acid solutions at several different concentrations were prepared in 0.2 M sodium acetate-acetic acid, pH 4.0, 4.5 and 5.5 buffers as described above.

Assay of PPO Activity and Inhibition

Enzyme activity was determined with and without oxalic acid using catechol (Sigma Chemical Co.) as substrate. The 3-mL standard reaction mixture contained 2.35

mL main buffer (0.1 M sodium acetate-acetic acid, pH 4.0, 4.5, 5.0, or 5.5), 0.3 mL oxalic acid prepared in the corresponding main buffer pH (described above) or control buffer (0.2 M sodium acetate-acetic acid, pH 4.0, 4.5, 5.0, or 5.5), 0.3 mL 0.5 M catechol in distilled water, and 0.05 mL PPO. The enzyme activity was measured by spectrophotometric determination of initial reaction rates at 420 nm and 25 °C with a Beckman Model DU 640 UV-Vis spectrophotometer. The pH of the reaction mixture was checked after each assay to confirm similar pH values for control and test systems. All assays were performed in duplicate each with 3 replications.

Preincubation of PPO with Oxalic Acid

Incubation was performed by mixing PPO with the main reaction buffer (0.1 M sodium acetate-acetic acid, pH 4.0, 4.5, 5.0, or 5.5) and the oxalic acid solutions prepared in the corresponding main buffer pH (described above) as in the standard reaction mixture. All samples were incubated for 30 min at room temperature (25 °C). Control samples contained the control buffer (0.2 M sodium acetate-acetic acid, pH 4.0, 4.5, 5.0, or 5.5) instead of oxalic acid. The residual PPO activities were measured immediately after the addition of catechol as described above.

Treatment of Oxalic Acid with Cupric Acetate

Cupric acetate stock solutions, 0, 7.5, 15, 30, 45, 60, and 75 mM, were prepared in 0.1 M sodium acetate-acetic acid, pH 5.0 buffer using cupric acetate monohydrate (Fisher Scientific, Fair Lawn, NJ). Oxalic acid was treated with cupric acetate as follows: 1.8 mL of oxalic acid final stock (0, 40, 60, or 100 mM) was mixed with 1.2 mL of cupric acetate stock (0, 7.5, 15, 30, 45, 60, or 75 mM) in test tubes. After incubation for 2 h at 25 °C, all mixtures were centrifuged for 15 min at 5000 x g and then assayed for PPO activity.

PPO activity was assayed after incubation with cupric acetate-treated oxalic acid for 1 h at 25 °C. The pre-incubation mixture consisted of 2.15 mL 0.1 M sodium acetate-acetic acid, pH 5.0 buffer, 0.5 mL cupric acetate-treated oxalic acid and 0.05 mL PPO. After incubation, the reaction was started by adding 0.3 mL of 0.5 M catechol. PPO activity was measured by spectrophotometric determination of initial reaction rates as described above. All assays were performed in duplicate each with 3 replications. Activities are expressed as % relative activity from the activity determined at pH 5.0 without cupric acetate and oxalic acid.

Restoration of Enzymatic Activity of Oxalic Acid-Treated PPO

The pre-incubation mixture consisted of 2.15 mL 0.1 M sodium acetate-acetic acid, pH 5.0 buffer, 0.3 mL oxalic acid final stock (40, 60, and 80 mM) and 0.05 mL PPO. After incubation for 1 h at 25 °C, the cupric acetate stocks (0.2 mL) were added to the reaction mixture. The reaction was started by adding 0.3 mL of 0.5 M catechol. PPO activity was measured as described above.

Scanning Studies

The scanning test mixtures containing 2.35 mL 0.1 M sodium acetate-acetic acid, pH 5.0 buffer, 0.3 mL oxalic acid (10, 40, 60, and 100 mM), and 0.05 mL PPO were incubated for 1 h at room temperature (25 °C). The control sample contained 0.2 M sodium acetate-acetic acid, pH 5.0 buffer instead of oxalic acid. The reaction was started with 0.3 mL of 0.5 M catechol and each scanned against the main buffer between 220-700 nm at 25 °C after 30 min reaction time at 25 °C. To investigate whether oxalic acid has any influence on changes in the absorption spectrum of the related pigmented oxidation products through pigment bleaching, the scanning test reaction mixture containing PPO and catechol was incubated for 30 min at 25 °C prior to addition of the

stock oxalic acid (100 mM). The reaction mixture immediately after the addition of the oxalic acid was scanned against the main buffer between 220-700 nm at intervals of 0, 5, 15, and 30 min. Control sample containing 0.2 M sodium acetate-acetic acid, pH 5.0 buffer instead of oxalic acid was always run parallel to the test sample. The reference sample contained ascorbic acid (1 %) instead of oxalic acid.

Inhibitory Effect of Dicarboxylic Acids

Glutaric acid was obtained from Aldrich Chemical Company (Milwaukee, WI) and succinic and malonic acids were obtained from Fluka (Switzerland). The 3-mL standard reaction mixture containing 0.1 M sodium acetate-acetic acid, pH 5.0 buffer, PPO, and dicarboxylic acid with the final concentrations of 8 or 16 mM was incubated for 30 min at 25 °C. The reaction was started with the addition of catechol and the residual PPO activity was measured as described above.

Results and Discussion

The pH Activity Profile of PPO

The activity of PPO toward catechol was investigated as a function of pH over the range 4.0-7.0 (Figure 3-1). PPO showed maximum activity at a pH around 5.5. About 80% activity, relative to that determined at pH 5.5, was detected at pH 5.0, but there was barely any activity at or above pH 6.0. The pH optima for apple PPO, reported to be dependent on cultivars and experimental factors used during the determination, range from 4.5-6.0 (Janovitz-Klapp and others 1989; Ferrar and Walker 1993; Zhou and others 1993).

Concentration- and pH-dependent Inhibitory Effect of Oxalic Acid

Inhibition of PPO by oxalic acid as a function of pH was studied at several different concentrations of oxalic acid and is shown in Figure 3-2. The extent of inhibition was

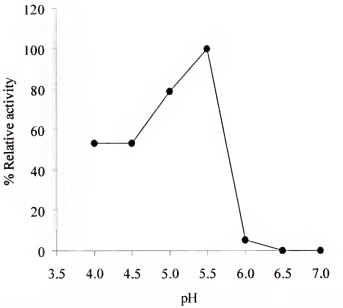


Figure 3-1. Effect of pH on the catalytic activity of PPO. PPO activity for the oxidation of catechol was determined in a standard reaction mixture buffered with 0.1 M sodium acetate, pH 4.0 to 5.5; and 0.1 M sodium phosphate, pH 6.0 to 7.0. (Standard deviations of all data were less than 0.05)

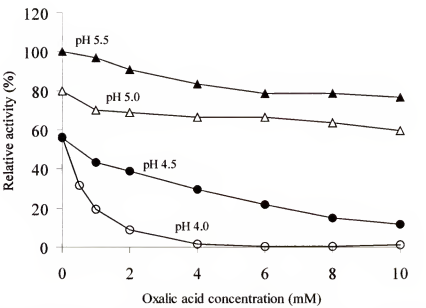


Figure 3-2. Effect of pH and oxalic acid concentration on the inhibition of PPO activity. Activities are expressed as % relative activity from the activity determined at pH 5.5 without oxalic acid. (Standard deviations of all data were less than 0.05)

influenced not only by oxalic acid concentration but also by pH changes. Inhibition was most prominent at pH 4.0 where approximately 60% enzyme activity was completely lost in the presence of 4 mM and higher oxalic acid concentrations. Although the enzyme exhibited comparable catalytic activity at pH 4.0 and 4.5, inhibition by oxalic acid on PPO was significantly lower at pH 4.5. The inhibition was much less evident at higher pH values (5.0 and 5.5) since PPO activity decreased slowly with increasing oxalic acid concentration (Figure 3-2).

Previous studies by Ferrar and Walker (1993) on Gala apple also demonstrated the pH dependence of PPO activity and its inhibition by oxalic acid. In their study, 3.3 mM oxalic acid did not show significant inhibition on Gala apple PPO between pH values of 4.7 to 6.0 (the optimal pH range for enzyme activity) and complete inhibition was observed near pH 4.0 where the enzyme showed about 60% relative activity in the absence of oxalic acid. Oxalic acid was also reported to exhibit higher inhibitory activity on sunflower PPO at pH 4.0 than at pH 6.0 (Marciano and others 1983). Unfortunately, they did not report the activity profile of sunflower PPO as a function of pH. Complete inhibition of spinach PPO, the activity of which was fairly constant at pH 5.5 to 6.8, occurred at pH 5.0, 5.5, and 6.0 in the presence of 1, 5, and 10 mM oxalic acid, respectively, and the extent of inhibition was small in the neutral region (pH 6.4 and 6.8) (Sato 1980a). The extent of inhibition by other carboxylic acids, such as benzoic and cinnamic acids, was also shown to be strongly pH-dependent, being greater at low pH (Robb and others 1966; Janovitz-Klapp and others 1990).

Since dissociation of oxalic acid is strongly dependent on pH (pKa values of oxalic acid: 1.2 and 4.2), the pH dependence of inhibition might reflect the effect of pH on the

relative amount of oxalic acid forms (monoanionic and dianionic species) in solution. Ferrar and Walker (1993) suggested that the monoanionic form of oxalic acid is responsible for inhibition since inhibition of Gala apple PPO predominates once the pH is reduced to near the pKa_2 of the inhibitor. Similar results were obtained with Red Delicious apples, inhibition being greater as pH decreases over the range of 4.0-5.5 (Figure 3-2). However, PPO activity of apple itself without oxalic acid was also strongly affected by pH changes as shown in this study (Figures 3-1 and 3-2) and by Ferrar and Walker (1993). Hence, an alternative hypothesis is that the pH dependence of inhibition could not be explained solely by changes in the relative amount of oxalic acid forms in solution but may also reflect the effect of pH on the overall charge of PPO protein or ionization state of its specific amino acid side chains (Yong and others 1990). Factors (*in vivo* and *in vitro*) that influence the pH optimum of PPO and, therefore, its potential activity may also influence the degree of inhibition by oxalic acid. The pH dependence of inhibition is complex (Figure 3-2) and further studies are necessary to clarify the inhibitory behavior of the oxalic acid forms (monoanionic and dianionic species) on PPOs in relation to the effect of pH changes on PPO protein.

Effect of Preincubation on Inhibition by Oxalic Acid

Figure 3-3 shows the effect of oxalic acid concentration at various pH conditions on the inhibition of apple PPO upon preincubation of the enzyme with the inhibitor for 30 min at 25 °C. Longer contact times with oxalic acid resulted in a significant reduction in enzyme activity, even at pH 5.0 and 5.5. The degree of inhibition was dependent on oxalic acid concentration and pH as also observed in inhibition studies without preincubation (Figure 3-2). Almost total loss of activity was achieved in the presence of 10 mM oxalic acid at pH 5.0 upon preincubation (Figure 3-3). Additionally, much

lower oxalic acid concentrations in the reaction resulted in a complete PPO inhibition upon incubation of the enzyme at pH 4.0 and 4.5. Since no reduction in activity was observed in the absence of oxalic acid (control) upon incubation, the increased inhibition was only due to the effect of oxalic acid on PPO over time. Such a preincubation effect on the inhibition of spinach PPO at neutral pH (6.8) with oxalic acid was also observed; while there was only about 5% reduction in enzyme activity without incubation, complete inhibition occurred following incubation of the enzyme with oxalic acid (2 mM) for 6 h at 25 °C (Sato 1980a). Since inhibition of spinach PPO also occurred at neutral pH where oxalic acid is fully in the dianionic state, Sato (1980a) suggested that the pH dependence of inhibition is not exclusively related to the occurrence of the monoanionic species in solution.

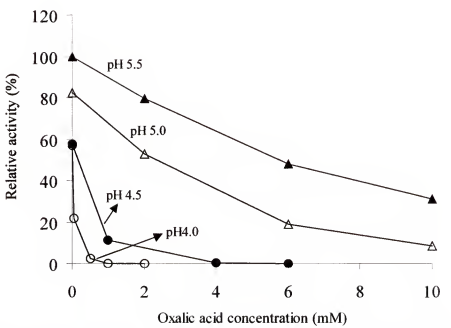


Figure 3-3. Effect of preincubation on the inhibition by oxalic acid for PPO at different pH values. PPO activity was measured after incubation with oxalic acid for 30 min at 25 °C. Activities are expressed as % relative activity from the activity determined at pH 5.5 without oxalic acid. (Standard deviations of all data were less than 0.05)

Inhibition Kinetics

Inhibition by oxalic acid on PPO was determined in the presence of several different concentrations of oxalic acid for 3 different fixed concentrations of catechol at pH 5.0 (Figure 3-4). Inhibition was characterized using Dixon and Cornish-Bowden plots (Dixon and Webb 1979) and established oxalic acid to be a noncompetitive inhibitor of PPO for the oxidation of catechol.

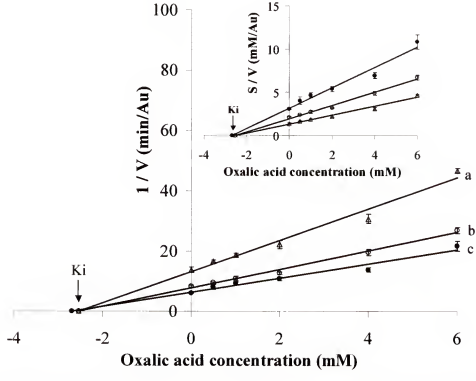


Figure 3-4. Kinetic determination of mode of inhibition. PPO activity was measured after incubation with oxalic acid for 30 min at 25 °C and pH 5.0 in a standard reaction mixture. Enzyme activity was measured in the presence of 10 (a), 25 (b), and 50 (c) mM catechol by spectrophotometric determination of initial reaction rate. Type of inhibition was determined by Dixon and Cornish-Bowden (insert) plots

Oxalic acid was found to be an effective inhibitor of PPO, with a K_i (inhibition constant) of 2.5 mM (Figure 3-4). Inhibition by oxalic acid could be based on the assumption that the enzyme is inactivated once the inhibitor binds and chelates the copper at the active site, whether or not substrate is also present. However, oxalic acid

was previously reported to demonstrate a mixed-competitive type inhibition on Gala apple PPO for the oxidation of 4-methyl catechol at pH 4.0, with a K_i of 0.55 mM (Ferrar and Walker 1993). Sato (1980a) also found oxalic acid to be a noncompetitive inhibitor of spinach PPO. Oxalic acid exhibited competitive inhibition on the catecholase reaction of mushroom PPO, with a K_i of 2.0 mM at pH 5.0 (Son and others 2000a) and with a K_i of 0.3 M at pH 5.6 (Yong and others 1990).

Much attention is given to the significant variations in physicochemical properties of the enzyme from different plant sources (Yoruk and Marshall 2003). Hence, PPOs from different sources may exhibit different types of inhibitory mechanisms in the presence of oxalic acid. Additionally, Kermasha and others (1993) related the variations in types of inhibition to differences in the conditions of the enzymatic assays; 2 acids, m-coumaric and ferulic, in the cinnamic acid series exhibited a mixed type inhibition with the spectrophotometric assay and a noncompetitive inhibition with the polarographic method. This may also be the case for apple PPO since Ferrar and Walker (1993) used the polarographic (oxygen uptake) method for the determination of enzymatic activity.

Chelating Effect of Oxalic Acid on the Active-Site Copper

In a chelator-metal complex, the chelator functions as a Lewis base (electron-donating species) and the metal ion acts as a Lewis acid (electron-accepting species) (Miller 1996). In order to determine whether oxalic acid is capable of forming stable complexes with free copper and, therefore, would act as a PPO inhibitor, it was treated with several different concentrations of cupric acetate and then tested for PPO inhibitory activity. The effect of copper-treated oxalic acid on PPO activity is shown in Figure 3-5.

Preincubation of oxalic acid with copper prior to assaying for PPO activity significantly reduced its inhibitory effect on the enzyme (Figure 3-5). Much higher levels

of PPO activity were detected with increasing copper concentrations in the system. No inhibition was observed in PPO activity in the presence of oxalic acid preincubated with 1/2-fold cupric acetate. The results suggest that with increasing copper concentration less free oxalic acid (unoccupied with copper) is available in the reaction mixture to inhibit PPO through chelation of the active site copper. Kahn and Andrawis (1985) reported that a 200- or 400-fold excess copper is required to significantly reduce the inhibitory effect of tropolone prior to assaying on mushroom PPO, suggesting that oxalic acid may have a higher binding constant to free copper than tropolone does.

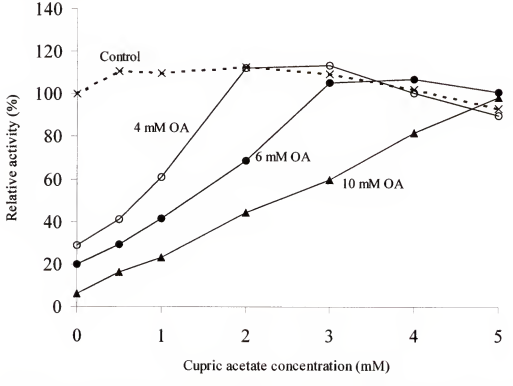


Figure 3-5. Effect of cupric acetate treated-oxalic acid on PPO activity. Oxalic acid (4, 6, and 10 mM) was treated with several different concentrations of cupric acetate as described in Materials and Methods. PPO activity was assayed after incubation with cupric acetate-treated oxalic acid for 1 h at 25 °C. Activities are expressed as % relative activity based on the activity determined at pH 5.0 without cupric acetate and oxalic acid. (Standard deviations of all data were less than 0.05)

It should be noted that the addition of cupric acetate to PPO as a control slightly affected the activity of the enzyme (Figure 3-5). Hence, the controls were always run parallel to test samples for comparison. PPO activity of the control initially increased by about 10 % in the presence of 0.5 to 3 mM cupric acetate, perhaps because of the existence of copper-free PPO in the enzyme preparation. However, in the presence of 5 mM cupric acetate, PPO activity of the control slightly decreased by 7%. Son and others (2000a), while studying the restoration of oxalic acid-treated mushroom PPO activity, observed a significant decrease (up to 45%) in the enzyme activity of the control with the addition of cupric chloride and explained the reason was due to the chloride ion, which was previously shown as a noncompetitive inhibitor of PPO by Janovitz-Klapp and others (1990). Added copper as cupric sulfate was shown to cause a more dramatic decrease in PPO activity, resulting in approximately 60% inhibition in the enzyme activity (Kahn and Andrawis 1985). Addition of cupric ions (as cupric sulfate, cupric chloride, and cupric nitrate) to a chelator-treated PPO preparation has been a widely used method for restoration of the enzyme activity (Sato 1980b; Kahn and Andrawis 1985; Son and others 2000a).

Further studies were conducted to determine the effect of added copper on the restoration of enzymatic activity to oxalic acid-inhibited PPO and are shown in Figure 3-6. PPO activity of oxalic acid-inhibited enzyme was recovered as a function of added copper concentration (as copper acetate) and resulted in complete restoration of the original activity. Sato (1980b) also observed full recovery of spinach PPO completely inhibited with ammonium oxalate by the addition of excess cupric ion. However, restoration of mushroom PPO activity inhibited with oxalic acid was less efficient,

resulting in about 60% maximum recovery of the original activity, when cupric ion was added (Sato 1980b; Son and others 2000a).

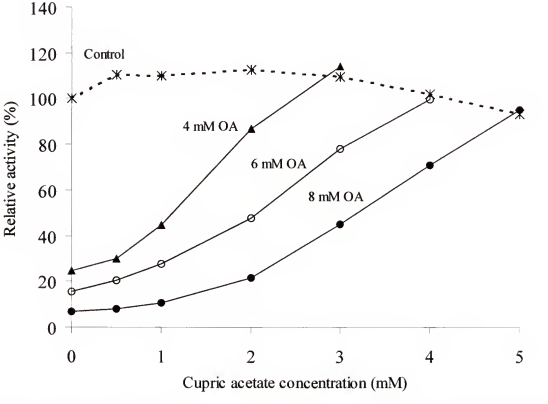


Figure 3-6. Effect of added copper, at a constant enzyme concentration, on the restoration of enzymatic activity to oxalic acid-treated PPO. PPO preparation was incubated with 4, 6, or 8 mM oxalic acid for 1 h at 25 °C and the enzyme activity was assayed with addition of various concentrations of cupric acetate. Activities are expressed as % relative activity based on the activity determined at pH 5.0 without cupric acetate and oxalic acid. (Standard deviations of all data were less than 0.05)

Son and others (2000a) also noted that the degree of observed recovery was dependent on oxalic acid concentration: the greater the inhibitor concentration, the less the observed recovery. The overall results of this study are in agreement with the findings of Sato (1980b) that inhibition of PPO by oxalic acid is due to its binding with copper at the active site of the enzyme to form an inactive complex. Yong and others (1990) reported that oxalic acid removes one copper atom from each binuclear site in PPO,

leaving the remainder as inactive mononuclear copper site, and addition of copper to the inactive enzyme preparation results in complete recovery of copper and catalytic activity. It thus seems that the inhibition exerted by oxalic acid is not caused by masking the active center but by the release of copper (Yong and others 1990).

Scanning Studies

The effect of oxalic acid on the absorption spectrum of the final product(s) of the PPO-catechol reaction was evaluated by UV-Vis scans. The spectra of the final product(s) obtained when a fixed amount of catechol was oxidized by a fixed amount of PPO preincubated with various concentrations of oxalic acid for 1 h are shown in Figure 3-7.

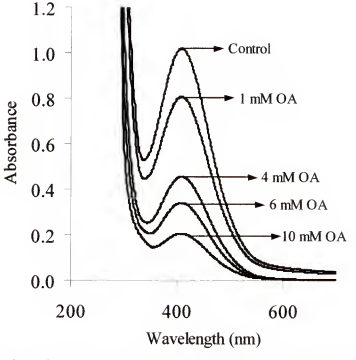
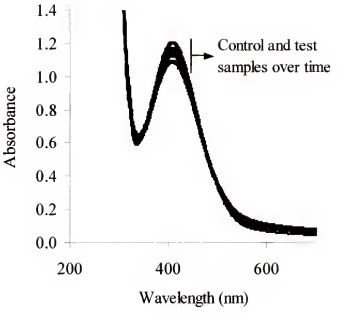


Figure 3-7. Effect of oxalic acid on the absorption spectrum of the final product(s) of PPO-catechol reaction. The numbers next to the arrows indicate oxalic acid concentrations tested (1, 4, 6, and 10 mM OA). The scanning reaction mixtures containing PPO and oxalic acid were incubated for 1 h at 25 °C at pH 5.0 prior to addition of catechol. All samples were scanned after a 30 min reaction time at 25 °C

The oxidized products had a maximum absorbance at a wavelength of 420 nm. This value is in agreement with the absorption range (395-475 nm) for the o-quinones reported by Interesse and others (1980). The change in absorbance at 420 nm is normally used for monitoring PPO activity based on the measurement of pigmented product formed from catechol (Zhou and others 1993; Son and others 2000a). Scanning studies showed that the ability of oxalic acid to diminish the quinone product formation was concentration-dependent (Figure 3-7). The level of the pigmented product(s) formed by evaluating the peak at 420 nm did show a decreasing trend with increasing oxalic acid concentration relative to the control. The higher level of product(s) was formed in the presence of 1 mM oxalic acid followed by 4 and 6 mM oxalic acid. In the presence of 10 mM oxalic acid a much greater decrease in the level of products was detected.

Additionally, incubation of oxalic acid (10 mM) with the final product(s) of PPO-catechol reaction demonstrated no influence in the level of the related pigmented oxidation products (Figure 3-8a). The curves representing the control and oxalic acid test samples were identical, regardless of the incubation time. However, when ascorbic acid replaced oxalic acid in the reaction, quinone bleaching was observed (Figure 3-8b). Ascorbic acid has the ability to change the absorption spectra by reducing the quinones back to the original colorless diphenols (McEvily and others 1992; Whitaker 1994). In order to determine whether oxalic acid acts on the enzyme and/or the reaction products, Son and others (2000a) compared the percent inhibition of oxalic acid on mushroom PPO by spectrophotometric (product formation) and polarographic (oxygen uptake) methods. In the presence of ascorbic acid, complete inhibition was detected in the rate of oxidation of catechol to colored products and there was no inhibition of oxygen uptake.

A)



B)

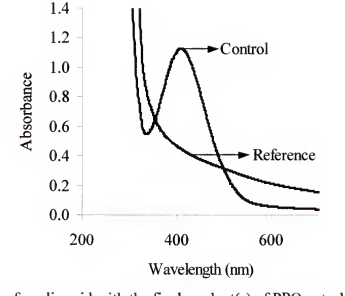


Figure 3-8. Incubation of oxalic acid with the final product(s) of PPO-catechol reaction. The scanning reaction mixture containing PPO and catechol was incubated for 30 min at 25 °C prior to addition of oxalic acid (10 mM). Test sample after addition of the oxalic acid was scanned as a function of time: 0, 5, 15, and 30 min (A). Control sample with no oxalic acid was always run parallel to the test sample and is shown for each incubation time interval (a). Reference sample (B) contained ascorbic acid instead of oxalic acid

However, they observed no significant difference in percent inhibition of oxalic acid on PPO determined by means of spectrophotometric and polarographic methods. This is in agreement with our results, indicating that oxalic acid acts on the enzyme by chelating the functional copper and, therefore, inhibits the formation of the products.

Comparison of Inhibitory Effectiveness of Dicarboxylic Acids

The relative inhibitory effect of oxalic acid on PPO was compared with other structurally related acids (dicarboxylic acids), including malonic, succinic, and glutaric acids. The results are listed in Table 3-1. None of them was found to be as effective as oxalic acid on PPO inhibition. At 8 and 16 mM concentrations, malonic acid showed slightly greater inhibition compared to glutaric and succinic acids. Malonic and succinic acids at the 2 mM concentration were also reported to be ineffective on spinach PPO (Sato 1980a). However, Pifferi and others (1974) reported that oxalic acid at pH 4.2 showed a lower inhibitory effect on PPO from sweet cherry fruit than malonic acid, but higher than succinic acid. Glutaric acid was not an efficient inhibitor. Son and others (2001) demonstrated oxalic acid as the most potent browning inhibitor among a number of carboxylic acids on fresh-cut apple slices.

It appears that the ability of oxalic acid to combine with the active-site copper and therefore inhibit PPO activity depends on its chemical structure and properties (Table 3-1). The inhibitory effectiveness of the dicarboxylic acids decreases with increasing chain length and dissociation constants, suggesting the possible role of steric interference in the interaction of the longer chains with the active site and of the degree of their ionization in solution. However, studies by Duckworth and Martin (2001) on surface complexation structures formed between hematite (surface hydroxyl groups of iron) and C_1-C_6 dicarboxylic acids demonstrated that a linear trend does not exist between the

carbon chain length of the ligands and the surface structures formed at pH 5.0. Oxalic, malonic, and glutaric acids formed bidentate surface complexation structures, whereas succinic and adipic acids formed monodentate structures. This was attributed to the variation of strain energies for the geometries of possible surface complexation structures (Duckworth and Martin 2001). Because there was a linear relationship between the binding constants and the second acidity constants (pK_2) for the bidentate ligands, it was suggested that as pK_2 decreases, the affinity of the second carboxylate functional group for the surface, and the overall stability of the complex increases linearly (Duckworth and Martin 2001). Oxalic acid was reported to be as effective an antibrowning agent as kojic acid and more potent than cysteine and glutathione on the catechol-mushroom PPO reaction (Son and others 2000a).

Table 3-1. Comparison of inhibitory effect of dicarboxylic acids on PPO activity [a]

Dicarboxylic acid	Chemical formula	Dissociation constants pK_1	Dissociation constants pK_2	% Inhibition [b] 8 mM	% Inhibition [b] 16 mM
Oxalic	$HOOC\text{-}COOH.2H_2O$	1.2	4.2	93.1	Nd [c]
Malonic	$HOOCCH_2COOH$	2.8	5.7	15.5	21.3
Succinic	$HOOC(CH_2)_2COOH$	4.1	5.6	5.1	7.1
Glutaric	$HOOC(CH_2)_3COOH$	4.3	5.4	10.3	16.4

[a] Enzyme activity was measured at 25 °C and pH 5.0. [b] The degree of inhibition on the reaction was expressed as percent inhibition, calculated using the formula [100(A-B)/A], where A and B were the enzyme activities in the control and test systems, respectively. [c] Nd, not determined

CHAPTER 4
MACHINE-VISION ANALYSIS OF ANTIBROWNING POTENCY FOR OXALIC ACID: A COMPARATIVE INVESTIGATION ON BANANA AND APPLE

Introduction

Bananas and apples are popular fruits in the world, with world production exceeding 35 and 85 million tons/yr, respectively (Nicolas and others 1994; Becker and others 2000). Bananas are one of the major crops in tropical and subtropical developing countries (Becker and others 2000). They are prone to enzymatic browning during handling and processing, and the fruit industry is interested in methods to prevent browning of minimally processed crops. Browning has a significant impact on the quality of bananas and apples, and their products, as it results in alteration of color, flavor, texture, and nutritional value (Sapers 1993; Nicolas and others 1994; Gooding and others 2001). Therefore, it is necessary to maintain the original color and quality of the fresh fruit during processing and in the marketplace.

Polyphenol oxidase (PPO; EC 1.10.3.1) causes enzymatic browning by catalyzing natural phenolic compounds to quinones (Nicolas and others 1994; Gooding and others 2001; Yoruk and Marshall 2003). Subsequent nonenzymatic oxidative polymerization reactions form brown, black or red pigments (melanins) (Sapers 1993; Yoruk and Marshall 2003). Many methods can be shown experimentally to diminish or prevent browning but are not all applicable for food use (McEvily and others 1992; Sapers 1993; Son and others 2001; Yoruk and Marshall 2003). PPO-catalyzed browning reaction can be prevented by means of eliminating from the reaction an active reaction element(s), that

is, enzyme, substrate(s), copper (prosthetic group) or a reaction intermediate (*o*-quinones) (Sapers 1993; Nicolas and others 1994; Yoruk and Marshall 2003).

Oxalic acid is a naturally occurring inhibitor of PPO in spinach (Sato 1980a, 1980b) and also was shown to inhibit the activities of PPOs from apple (Ferrar and Walker 1993; Chapter 3), sweet cherry (Pifferi and others 1974) and mushroom (Yong and others 1990). Inhibition of PPO by oxalic acid was previously shown due to its binding with the active site copper to form an inactive complex (Sato 1980b; Yong and others 1990; Chapter 3). The inhibition mode was noncompetitive for Red Delicious apple PPO (Chapter 3) and mixed competitive for PPO from Gala apples (Ferrar and Walker 1993). Although it is not an approved food additive, oxalic acid is a natural component of a large number of plants such as asparagus, broccoli, Brussels sprouts, carrot, garlic, lettuce, onion, parsley, pea, potato, purslane, radish, spinach, tomato, and turnip (USDA 1984).

There is little, if any, documentation on the potential applicability of oxalic acid in prevention of browning in minimally processed banana. There are few reports on antibrowning effect of oxalic acid on Liberty apples (Son and others 2000b; 2001) and no recent study on the effect on Red Delicious apples. The extent of browning varies greatly among apple cultivars (Coseteng and Lee 1987; Sapers and Douglas 1987). Factors responsible for this were reported to be differences in PPO activity and the substrate content (Coseteng and Lee 1987). Red Delicious apple was previously shown to have the highest PPO activity among many apple cultivars (Janovitz-Klapp and others 1989). The objectives of this study were, therefore, to investigate the antibrowning effectiveness of oxalic acid on fresh-cut surfaces of banana and Red Delicious apple and to compare its

antibrowning effectiveness to some other structurally related carboxylic acids such as malonic, succinic, and glutaric acids using a machine-vision system. Machine vision, a non-destructive method, can rapidly quantify the disappearance of lighter colors and the formation of darker colors in non-uniformly colored surfaces of many foods (Luzuriaga and others 1997; Luzuriaga 1999).

Materials and Methods

Preparation of Fruit Slices and Their Treatments with Oxalic Acid

Oxalic acid solutions were prepared in double de-ionized water using oxalic acid dihydrate (Acros, New Jersey, U.S.A.). Bananas and apples (Red Delicious), obtained from a local grocery store, were immediately processed and subjected to color analysis. The fruit samples peeled with a stainless steel knife were sliced (1 cm thick) using a stainless steel hand slicer. Banana slices were dipped in oxalic acid solutions (500 mL) at concentrations of 10, 20, 40, 60, 80, and 100 mM for 10 min. Apple slices were dipped in solutions (500 mL) containing oxalic acid at concentrations of 3, 5, 10, 20, and 40 mM for 10 min. Control slices were dipped in double de-ionized water for 10 min. Excess surface liquid was removed by blotting with cheesecloth.

Video images of samples were captured in a light box (Luzuriaga and others 1997) immediately after treatment (at 0 h) and every 1 h intervals during storage at room temperature (23 °C) for 5 h for banana and 7 h for apples. Samples were stored on a plastic screen mesh in a closed container containing tap water leveled just under the screen to minimize drying of samples during storage. Four slices were analyzed for each treatment. The experiment was repeated using a different batch of banana and apple about 1 month later.

Comparison of Antibrowning Effectiveness of Dicarboxylic Acids

Glutaric acid was obtained from Aldrich Chemical Company (Milwaukee, Wis.), and succinic and malonic acids from Fluka (Switzerland). The 100 and 10 mM solutions were prepared in double de-ionized water for banana and apple slices, respectively. Samples were dipped in these solutions (500 mL) for 10 min. Control slices were dipped in double de-ionized water. Video images of the samples were captured at 1 h intervals, as described above. Four slices were analyzed for each treatment. The experiment was repeated using a different batch of banana and apple, 2 weeks later.

Color Data Analysis

Browning inhibition was analyzed using a machine-vision system with D65 illumination (Luzuriaga and others 1997; Luzuriaga 1999). Average L* values of the pixels representing the total surface area of each banana and apple slice (4 replicates for each treatment level) were analyzed to assess the extent of browning using Color Expert software (Engineering & Cyber Solutions, 2003). L* parameter ranges from white (100) to black (0). Browning on cut surfaces was evaluated based on the experimental parameters, oxalic acid concentration and storage time, using two approaches:

- Observing trends in $L^*_0 - L^*_t$ values, calculated by subtracting the values obtained immediately before storage (L^*_0) from the values obtained after a storage time t (L^*_t).
- Comparing the percent differences (browning index) between the controls and treatments ΔL^* values after a specified storage time t, calculated according to Sapers and Douglas (1987):

$$\text{Browning index} = \frac{\Delta L^*\text{control} - \Delta L^*\text{treatment}}{\Delta L^*\text{control}} \times 100 \quad (1)$$

where ΔL^* is the difference between L* values at indicated storage time and values at 0 h. Browning index values are between 0 and 100, and signify the extent of effective browning inhibition of a treatment (Sapers and Douglas 1987).

Color Spectra

Temporal changes in color spectra of control and treatments on apple and banana slices were compared using the Color Expert software (Engineering & Cyber Solutions, 2003). Color was represented in the 512-block RGB system. The R, G, and B axes are divided into 8 regions, each. Any color that falls into one of the resulting 512 blocks is represented by the center color of the block. The average colors of the 4 replicates at each treatment level, corrected by area, were calculated. The color block that changed more than 5% over storage time were selected and compared.

Statistical Analysis

Color data were analyzed in MINITAB statistical software using one-way analysis of variance (ANOVA). ANOVA's were calculated at every time step. Comparison of means was done using the least significant difference (LSD) test.

Determination of pH and Oxalic Acid Concentration in the Treated Tissues

Oxalic acid treated banana tissue was macerated in two-fold (w/w) double de-ionized water in a Waring blender. The homogenate was filtered through 8 layers of cheesecloth and the pH of the filtrate was determined using a digital pH meter. Treated apple tissues were macerated without addition of water and pH was determined similarly after filtering through cheesecloth. An oxalate kit (Oxalate Urinalysis Diagnostic Kit: Procedure Number 591, Sigma, St. Louis, M.O.) was used to determine tissue concentration of oxalic acid in treated bananas and apples. This is an enzymatic method based on the oxidation of oxalic acid by oxalate oxidase followed by measurement of hydrogen peroxide produced during the reaction by a peroxidase-catalyzed reaction. The procedure described by Ferrar and Walker (1993) was used for preparation of the samples for oxalic acid analysis. Treated banana and apple tissues, homogenized in 4-fold and

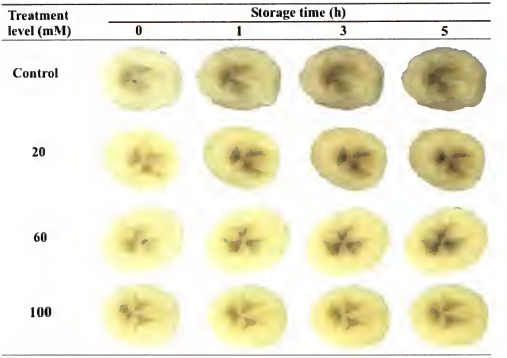
1-fold (w/w) 0.2 M KH_2PO_4/Na_2HPO_4, pH 6.0 buffer, respectively, were centrifuged at 10000 x g for 15 min. Fractions (5 mL) of the supernatant were then assayed for oxalic acid. Analyses were performed in triplicates.

Results and Discussion

Visual Observation

Video images of banana and apple slices dipped in different concentrations of oxalic acid are shown in Figure 4-1. Immediately after treatment (at 0 h), oxalic acid dipped-samples were slightly lighter than the water-dipped controls, regardless of the oxalic acid concentration used. This might be due to browning inhibition by oxalic acid during the dipping period, and there may also be some pigment bleaching. However, in a PPO-catechol reaction model system, incubation of oxalic acid (10 mM) with the final product(s) of the reaction demonstrated no influence in the level of the related pigmented oxidation products (Chapter 3). The best color preservation for the sliced bananas was obtained with 60 and 100 mM oxalic acid solutions during the 5 h storage period. Compared with controls, these treatments showed significantly less browning although gradual discoloration was noticed at the center of the slice (Figure 4-1). The center of the sliced banana normally turns brown much more rapidly than the peripheral pulp due to the localization of PPO mainly in this area (Galeazzi and others 1981).

For apple, treatments consisting of 3 mM oxalic acid failed to inhibit browning and the samples were slightly lighter than the controls (Figure 4-1). Oxalic acid solutions of 5 and 10 mM were effective in browning inhibition in sliced apple, showing essentially no color change to the naked eye during the 7 h storage period.



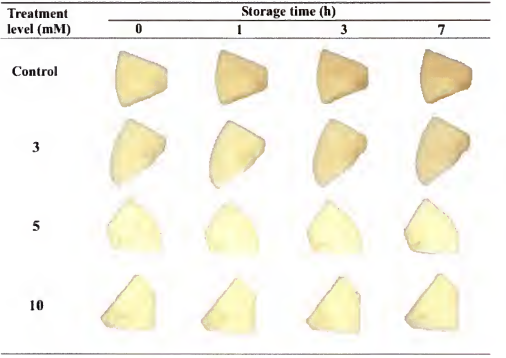


Figure 4-1. Video images of oxalic acid treated banana and apple slices during storage. Control samples were dipped in water

The effect of oxalic acid on color preservation of sliced banana and apple was evaluated quantitatively using the machine-vision system. Average L* values obtained from the images of each oxalic acid treated fruit slice were analyzed to assess the extent of browning using the Color Expert software. An observed decrease in L* value is indicative of browning, and is commonly used in browning indices (Sapers and Douglas 1987; Molnar-Perl and Friedman 1990; Lozano-De-Gonzalez and others 1993; Sapers and Miller 1993; Son and others 2000b, 2001).

Effect of Oxalic Acid and Storage Time

Change in browning (L^*_0–L^*_t) of banana slices treated in solutions containing oxalic acid at concentrations of 0 to 100 mM is shown in Figure 4-2. The intensity of browning on the surfaces was evaluated based on the experimental variables (oxalic acid concentration and storage time) and the reproducibility between the replications in overall trend between the experiments (Figure 4-2). Browning inhibition attained with 10 and 20 mM oxalic acid treatments was minor as the observed increase in (L^*_0–L^*_t) indicates progressive browning of the sliced banana. Dipping the slices in solutions containing 40 mM oxalic acid significantly reduced browning ($p<0.05$) during 5 h storage period. However, the best color preservation was obtained with oxalic acid concentrations of 60 mM and higher during the 5 h storage period as reflected by the color parameter (Figure 4-2) and visual observations (Figure 4-1). Additionally, there was a good reproducibility between replicate experiments (Figure 4-2). No significant difference was observed in browning inhibition potency of these latter treatments at any time interval ($p<0.05$). Browning inhibition achieved with treatments of 60 mM and higher oxalic acid on banana slices was evident although some discoloration was observed at the center of the fruit slices (Figure 4-1).

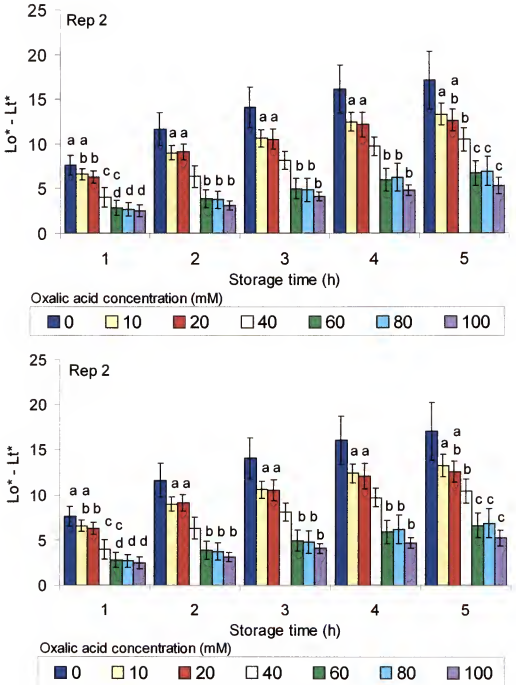


Figure 4-2. Changes in browning (L_0^*-L_t^*) on cut surfaces of banana as a function of oxalic acid concentration and storage time. LSD tests at $\alpha = 0.05$ were performed for treatments at each interval time. Treatments not significantly different from one another are shown with the same letter on the bar graphs

The color data were also analyzed using browning index approach with repeated measures as recommended by Sapers and Douglas (1987) and is shown in Figure 4-3. This method in which the degree of browning in treated samples is compared with that of the corresponding control samples on a percent basis is often used to evaluate potency of a browning inhibitor (Sapers and Douglas 1987; Molnar-Perl and Friedman 1990; Lozano-De-Gonzalez and others 1993; Sapers and Miller 1993). The results demonstrated that browning index trends of Rep 1 and Rep 2 were comparable (Figure 4-3). A slight variation in the level of browning indices between the experiments is attributable to fruit-to-fruit variability (Figure 4-2). This may result from seasonal variation of PPO activity and concentration (units/g pulp) for bananas (Jayaraman and others 1982). Changes in marketing and environmental conditions during ripening of the fruit may be plausible explanations for the variability in degree of enzymatic browning and its inhibition (Jayaraman and others 1982; Barrett and others 1991; Nicolas and others 1994).

Under the conditions of this study, browning index values of 60 and higher indicated that the treatment was highly effective in controlling browning of banana slices as judged by the visual evaluations of the samples (Figures 4-3 and 4-1). The 10 and 20 mM oxalic acid solutions were not effective in controlling browning (Figure 4-3). However, an increasing trend in browning index was observed with increasing oxalic acid concentration. Based on the trends from Figures 4-2 and 4-3, the best browning inhibition during the 5 h storage period was achieved with 60 mM and higher oxalic acid treatments.

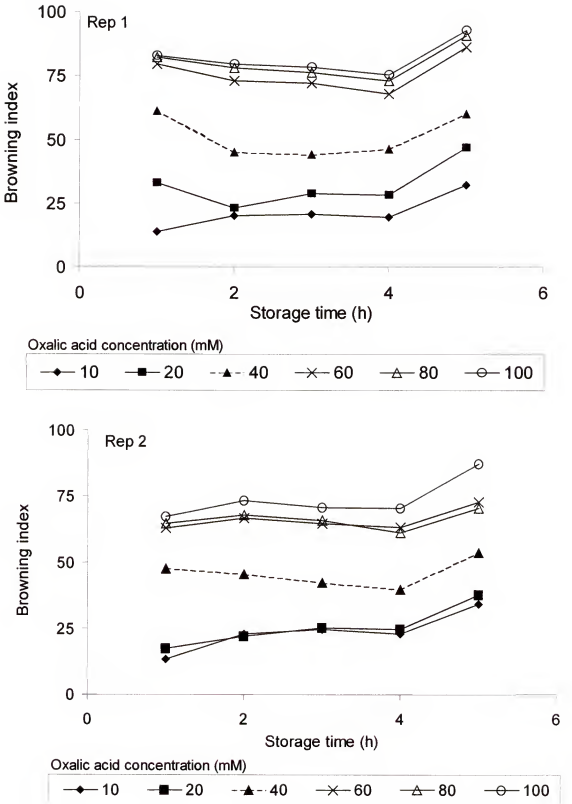


Figure 4-3. Browning indices on cut surfaces of oxalic acid treated banana

Change in browning of apple slices treated with solutions containing oxalic acid at concentrations of 0 to 40 mM is shown in Figure 4-4. Browning inhibition of sliced apple was attained with relatively lower concentrations of oxalic acid compared to sliced banana (Figure 4-2). For sliced apples, treatments containing 3 mM oxalic acid were ineffective, resulting in gradual increase in darkening of the surfaces during the 7 h storage period (Figures 4-4 and 4-1). The most effective color preservation was obtained with treatments containing 5 mM and higher oxalic acid during the 7 h storage period (Figure 4-4). There was no significant difference in relative antibrowning potency of these treatments during 5 h storage period ($p<0.05$) and little variation was noticed at 7 h storage period in Rep 1. The minimum concentrations of oxalic acid in solutions and in rhubarb juice to effectively suppress browning of Liberty apples for up to 3 h storage at room temperature were 0.05 and 0.07%, respectively (Son and others 2000b, 2001)

Browning indices for apple slices are shown in Figure 4-5. A positive correlation between browning indices and oxalic acid concentration was evident. Browning index values of 50 and higher indicated significant browning inhibition for sliced apple. Differing degrees of browning inhibition both between and within cultivars was attributed to apple-to-apple variability (Coseteng and Lee 1987; Sapers and Douglas 1987), which could account for differences in PPO activity and polyphenol content (Coseteng and Lee 1987; Sapers and Douglas 1987; Janovitz-Klapp and others 1989; Barrett and others 1991; Nicolas and others 1994). Browning indices on the sliced apple obtained with treatments containing 5 mM and higher oxalic acid solutions showed similar effectiveness during 7 h storage period and were noticeably higher compared to those obtained with the 3 mM treatment (Figure 4-5).

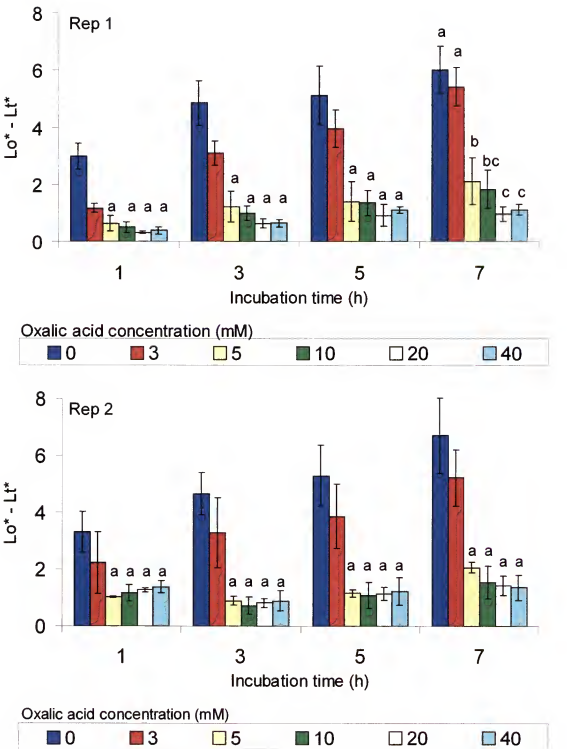


Figure 4-4. Changes in browning (L_0^*-L_t^*) on cut surfaces of apple as a function of oxalic acid concentration and storage time. LSD tests at $\alpha = 0.05$ were performed for treatments at each interval time. Treatments not significantly different from one another are shown with the same letter on the bar graphs

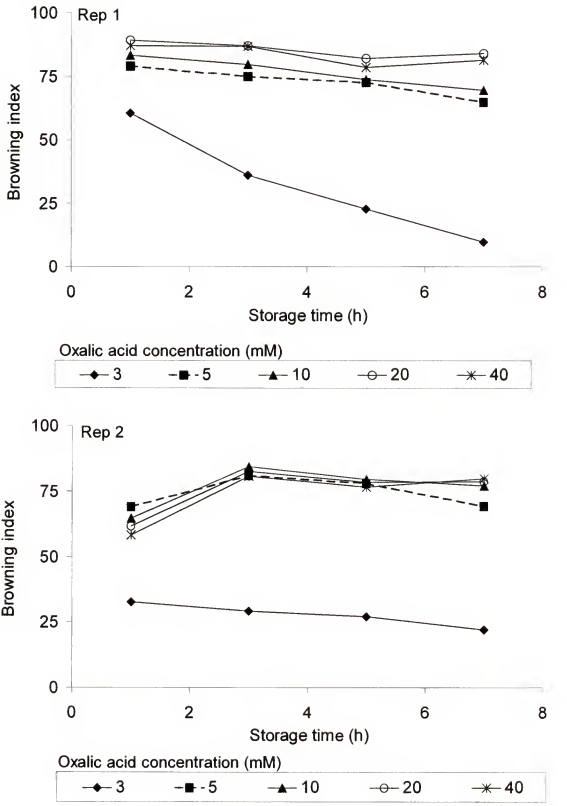


Figure 4-5. Browning indices on cut surfaces of oxalic acid treated apple

Changes in Color Spectra

An alternative use of the machine-vision system is to analyze temporal changes in color spectra of a sample, providing additional insight into quantitative changes of color during browning. Changes in color spectra of sliced banana are shown in Figure 4-6. For water-dipped samples (controls), a sharp decrease in the level of lighter colors (color blocks 498 to 510) followed formation of darker colors (color blocks 436 and higher) after 1 h storage (Figure 4-6). Disappearance of lighter colors was relatively slow for banana samples treated with 20 mM oxalic acid solutions compared to the control samples. However, there was practically no reduction in the level of the lighter colors on the slices treated with 60 mM and 100 mM oxalic acid solutions (Figure 4-6). The 100 mM oxalic acid treatments showed slightly higher tendency to preserve the original lighter colors. Additionally, formation of darker colors over the storage period was less evident on the 60 and 100 mM oxalic acid treatments. This is consistent with interpretation of color data shown in Figures 4-2 and 4-3 and with visual observations (Figure 4-1) that oxalic acid effectively reduced darkening of sliced banana at concentrations of 60 mM and higher.

Similarly, color spectrum of the water-dipped apples (controls) was drastically changed during the storage period (Figure 4-7). A sharp decrease in the level of the lighter colors (color blocks 501 to 509) followed an increase in the level of darker colors (color blocks 500 and higher) for control samples after 3 h storage. There was no noticeable difference between controls and the 3 mM oxalic acid treatments in terms of formation of darker colors and disappearance of the lighter colors. The most effective color preservation was obtained with treatments consisting of 5 mM and 10 mM oxalic acid. The color spectra of these apple samples were comparable, regardless of the storage

time period (Figure 4-7). The overall results support the hypothesis that oxalic acid treated apple slices exhibit its "natural" color, and therefore oxalic acid has potential to prevent enzymatic browning.

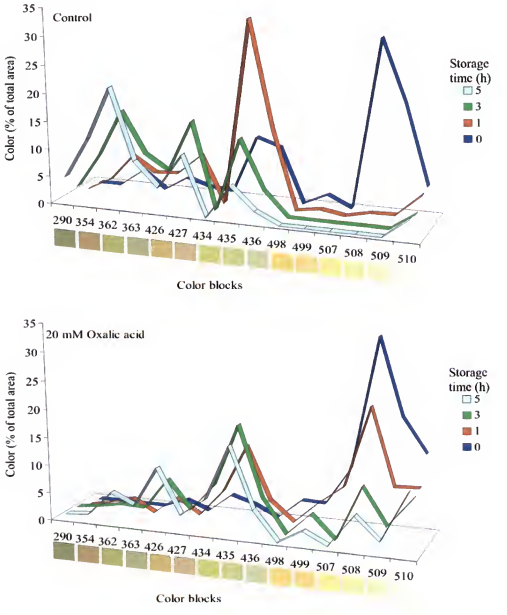
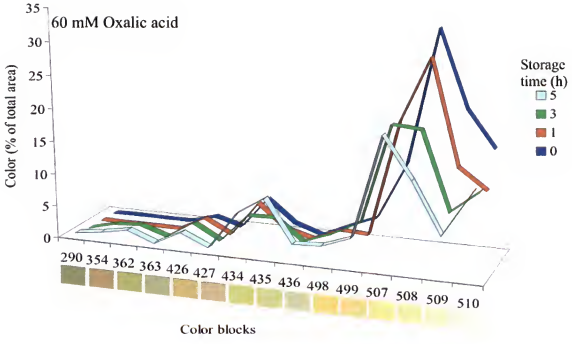


Figure 4-6. Temporal changes in color spectra on cut surfaces of oxalic acid treated banana. Control samples were dipped in water. Color was represented in the 512-block RGB system



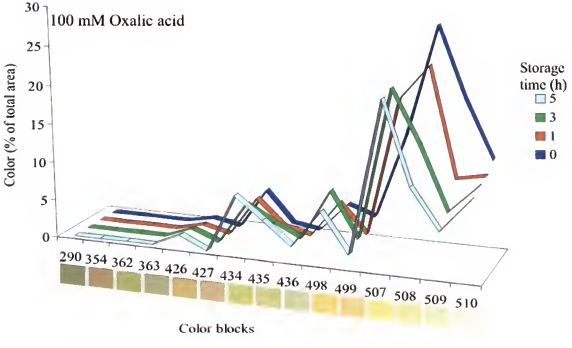


Figure 4-6 Continued

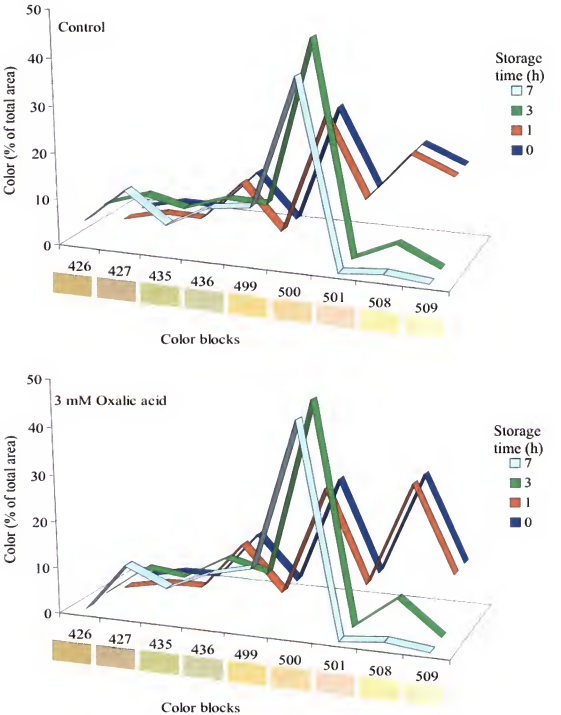


Figure 4-7. Temporal changes in color spectra on cut surfaces of oxalic acid treated apple. Control samples were dipped in water. Color was represented in the 512-block RGB system

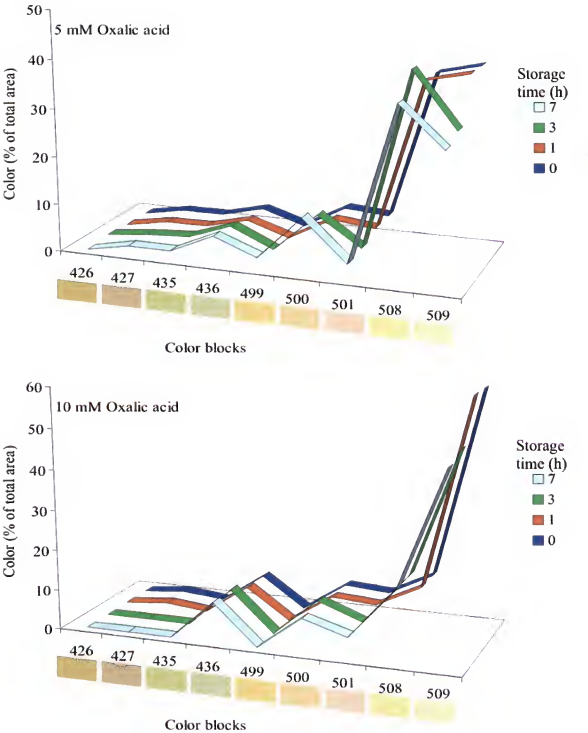


Figure 4-7 Continued

The pH and Oxalic Acid Concentration

Ferrar and Walker (1993) demonstrated that a large concentration of oxalic acid (1700 mg/100g of fresh material) produced by the phytopathogen *Sclerotinium rolfsii* in bean pods caused a dramatic reduction of tissue pH, while oxalic acid (90 mg/100 g of fresh material) produced in apples had no significant effect on tissue pH. Hence, the pH and the tissue concentration of oxalic acid in the treated banana and apple slices were evaluated and given in Table 4-1. It is apparent that the level of oxalic acid used for browning inhibition had little effect on the overall tissue pH of both bananas and apples. Analyses of oxalic acid levels in the treated banana and apple tissues indicated that as the treatment level increased the average concentration of oxalic acid increased in the tissues, as expected (Table 4-1).

Table 4-1. Change in pH with oxalic acid concentration in the treated tissues

Fruit	Treatment level (mM)	Δ pH [a]	Average concentration of oxalic acid (mg / g fresh wt tissue) [b]
Banana	20	0.1	0.30 ± 0.09
	40	0.1	0.53 ± 0.11
	60	0.2	0.85 ± 0.12
	80	0.3	1.29 ± 0.11
	100	0.3	1.65 ± 0.04
Apple	3	0.1	0.06 ± 0.01
	5	0.2	0.10 ± 0.04
	10	0.2	0.19 ± 0.05

[a] Δ pH = pH control (water dip) – pH oxalic acid treated tissue. [b] Average oxalic acid dihydrate concentration

There was a linear relationship (R^2=0.99) between treatment levels and total oxalic acid values measured in the tissues of sliced banana (Table 4-1). Concentration of average oxalic acid dihydrate in the sliced banana (100 g) for a significant reduction in browning inhibition was 85 mg. However, the minimum concentration of oxalic acid dihydrate in the treated apple tissues (100 g) for an effective antibrowning activity was 10

mg. Oxalic acid was also shown to suppress browning of Liberty apple cultivar at low concentrations (1.6-2.5 mg/100 g fresh material), where fresh-cut apple slices were treated with 0.05% oxalic acid solution, for 3 h storage at room temperature (Son and others 2001). Notice that oxalic acid used in this investigation on browning inhibition of Red Delicious apples was in the form of oxalic acid dihydrate. Our results indicated that oxalic acid has potential to prevent browning of the sliced apples for longer storage periods (Figures 4-4, 4-5, and 4-7).

Oxalic acid in both soluble and insoluble forms is a natural component of many higher plants and it was suggested to have some selective, functional significance or adaptation during normal plant development (Ilarslan and others 1997). A number of foods from plant origin contain relatively large amounts of oxalic acid (mg/100 g of fresh material), including asparagus (130), broccoli (190), Brussels sprouts (360), cabbage (100), cucumbers (20), eggplant (190), garlic (360), lettuce (330), purslane (1310), spinach (970), tomato (50), and turnip (210) (USDA 1984). The daily consumption of oxalic acid in Western diet was reported to be within the range of 70 to 150 mg (Son and others 2001). Oxalic acid at high concentrations has a tendency to reduce magnesium and calcium bioavailability in humans (Prenen and others 1984; USDA 1984). It was, however, reported that these oxalic acid rich foods usually containing sufficient magnesium or calcium would not interfere with the magnesium and calcium availability of other foods in the diet (USDA 1984).

Oxalic acid was shown to effectively suppress browning of Liberty apples by Son and others (2001) and Red Delicious apples in this study at concentrations well below the level found naturally in such plants listed above. However, the minimum average

concentration of oxalic acid dihydrate (85 mg/ 100 g of fresh material) in the treated banana tissues to diminish browning is relatively higher than that in the sliced apple cultivars (Table 4-1). This level of oxalic acid was still less than the level naturally present in such plants as spinach, lettuce and Brussels sprouts, whereas it was higher than that found in cucumbers, onion, okra, parsnip and squash (USDA 1984). The overall results suggest that there may be a possibility of using oxalic acid to control browning of minimally processed apples. If oxalic acid were used to control browning on sliced banana, magnesium and/or calcium supplementation may be required. However, further studies are needed to address the nutritional consequences of the levels used in these commodities.

Comparison of Antibrowning Effectiveness of Dicarboxylic Acids

The relative antibrowning potency of oxalic acid on sliced bananas and apples was compared to other structurally related acids (dicarboxylic acids), including malonic, glutaric and succinic acids and the results are shown in Figure 4-8. None of the alternative acids was found to be as effective an antibrowning agent as oxalic acid. There was no significant difference in browning inhibition of the sliced banana achieved with malonic, glutaric and succinic acids ($p<0.05$). Moreover, these latter acids did not act as browning inhibitors on apple slices; rather they enhanced browning (Figure 4-8). There was no significant difference in the extent of browning among these treatments ($p<0.05$).

Son and others (2001) also reported oxalic acid as the most potent browning inhibitor among a number of carboxylic acids on fresh-cut Liberty apples. They grouped the carboxylic acid into 3 categories, the best, medium, and weak inhibitors, based on their antibrowning activity. Oxalic and malonic acids was categorized in the best inhibitor group whereas succinic acid was in the weak inhibitor group (Son and others

2001). The potential differences in the nature of Liberty and Red Delicious apple cultivars, i.e. texture, polyphenol content, PPO activity level and physiochemical properties of the enzyme (Coseteng and Lee 1987; Janovitz-Klapp and others 1989; Ferrar and Walker 1993) may be plausible explanations for these disparities.

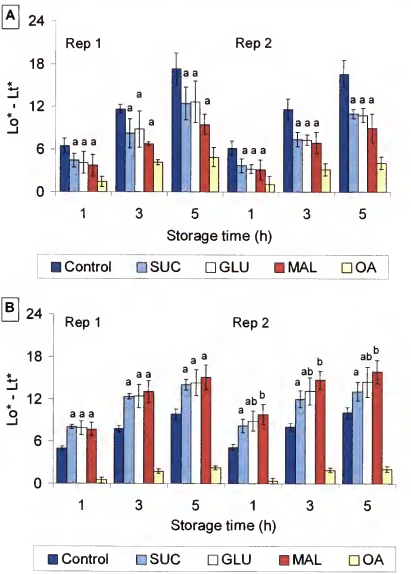


Figure 4-8. Comparison of antibrowning effectiveness of dicarboxylic acids on banana and apple slices. Banana (A); apple (B); OA; oxalic acid, MAL; malonic acid, GLU; glutaric acid, SUC; succinic acid. Control samples were dipped in water. LSD tests at $\alpha = 0.05$ were performed for treatments at each interval time. Treatments not significantly different from one another are shown with the same letter on the bar graphs

The previous studies in our laboratory (see Chapter 3) compared the inhibitory effect of oxalic acid on PPO from Red delicious apple to malonic, glutaric, and succinic acids in a reaction model system and found oxalic acid as the most potent inhibitor of the enzyme. It was suggested that inhibitory effectiveness of the dicarboxylic acids decreases with increasing chain length and dissociation constants, signifying the possible role of steric interference in the interaction of the longer chains with the enzyme and of the degree of their ionization in solution (Chapter 3). It was concluded that the ability of oxalic acid to inhibit browning depends on its chemical structure and properties. Malonic and succinic acids were also reported to be ineffective in inhibition of spinach PPO (Sato 1980a). However, Pifferi and others (1974) reported that oxalic acid shows lower inhibition on PPO from sweet cherry fruit than malonic acid but higher than succinic acid, and glutaric acid was not an efficient inhibitor at all.

When temporal changes in color spectra were analyzed for sliced apples treated with malonic, glutaric and succinic acid solutions (Figure 4-9), the level of the darker colors formed (particularly color blocks 354 to 427) during the 5 h storage period was relatively higher for these treatments than for the water-dipped controls. The results suggested that quantification of temporal changes in color spectra could be an alternative way of determining the effectiveness of a treatment on food color (Figures 4-6, 4-7, and Figure 4-9).

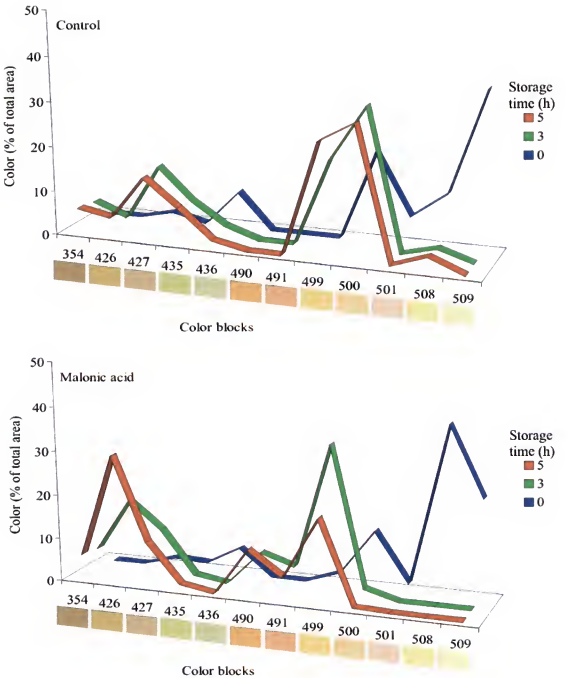


Figure 4-9. Changes in color spectra during browning of apple treated with dicarboxylic acids. Apple slices were treated with 10 mM of malonic, glutaric, and succinic acids. Control samples were dipped in water. Color was represented in the 512-block RGB system

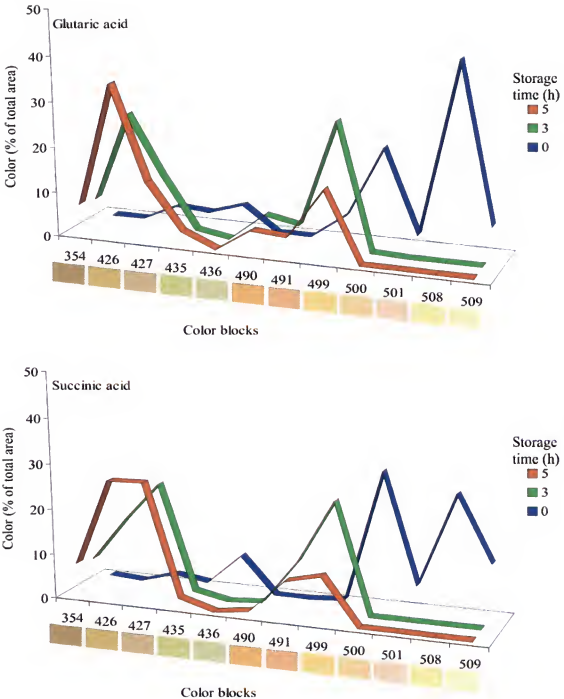


Figure 4-9 Continued

CHAPTER 5
INVESTIGATIONS ON POTENTIAL OF OXALIC AND ASCORBIC ACIDS TO CONTROL BROWNING OF MINIMALLY PROCESSED APPLE AND BANANA USING MACHINE VISION

Introduction

Apples and bananas are extremely prone to enzymatic browning during handling and processing (Nicolas and others 1994; Gooding and others 2001). Enzymatic browning occurs as a result of oxidation of phenolic compounds to quinones by polyphenol oxidase (PPO) and their eventual (nonenzyme-catalyzed) polymerization to melanin pigments (Vamos-Vigyazo 1981; Sapers 1993; Nicolas and others 1994; Yoruk and Marshall 2003). These biochemical reactions, also proceeding in several other fruits and vegetables, cause deterioration in food quality by changing nutritional and organoleptic properties (Vamos-Vigyazo 1981; Yoruk and Marshall 2003). Owing to its significant economic impact to the fruit and vegetable industry (McEvily and others 1992; Sapers 1993; Martinez and Whitaker 1995), control of adverse browning in plant products has received particular attention from researchers all over the world.

Oxalic acid was shown to effectively suppress browning of Liberty and Red Delicious apples (Son and others 2001; Chapter 4) at concentrations well below the level found naturally in such commodities as spinach, parsley, carrot, turnip etc. (Table 2-2). Average concentration of oxalic acid (mg/100 g of fresh material) in Liberty apple slices treated with 0.05% oxalic acid solution was 1.6-2.5 (Son and others 2001) and that of oxalic acid dihydrate in Red Delicious apples treated with 5 mM oxalic acid solution was 10 (Chapter 4). Browning inhibition was most prominent on banana slices treated with

oxalic acid solutions at concentrations as low as 60 mM where the concentration of oxalic acid dihydrate in the treated tissues was 85 mg/100 g of fresh material (Chapter 4).

There is substantial interest in using ascorbic acid along with other inhibitors for better control of enzymatic browning in the manufacturing of fruit juices, purees, fresh and frozen sliced fruits, and canned fruits and vegetables (Mcevily and others 1992). For example, a combination of ascorbic acid and citric acid was found to be more effective than ascorbic acid alone (Sapers and others 1989). This is probably due to the stability of ascorbic acid in an acidic environment and the inhibition effect of the acidic environment on the catalytic activity of PPO (Yoruk and Marshall 2003). Ascorbic acid inhibits melanin formation by preventing the accumulation of *o*-quinones, or they may form stable colorless products (Mcevily and others 1992; Sapers 1993; Nicolas and others 1994). Citric acid may also function as PPO inhibitor through its chelating action (Mcevily and others 1992; Sapers 1993). Inhibition of PPO by oxalic acid was previously shown due to its chelating ability with the active site copper (Sato 1980; Son and others 2000a; Chapter 3). Hence, the objective of this study was to extend the previous knowledge gained by investigating the effect of minimal concentrations of oxalic acid in conjunction with ascorbic acid on browning inhibition of apple (Red Delicious) and banana slices using a machine-vision system. Samples with non-uniform color and/or surface can be evaluated objectively in this method (Luzuriaga 1999; Chapter 4).

Materials and Methods

Preparation of Fruit Slices and Their Treatments with Oxalic and Ascorbic Acids

Solutions containing oxalic acid dihydrate (Acros, N.J.), ascorbic acid (Fisher Scientific, Fair Lawn, N.J.), and oxalic acid dihydrate plus ascorbic acid were prepared in double de-ionized water. Apples (Red Delicious) and bananas, obtained from a local

grocery store, were immediately processed and subject to color analysis. The fruit samples peeled with a stainless steel knife were sliced (1 cm thick) using a stainless steel hand slicer. Fruit slices were dipped in 3 different combinations of oxalic acid (1.5, 3, and 5 mM for apple and 10, 20, and 40 mM for banana) with 0.5% ascorbic acid for 10 min. Control samples dipped in water, ascorbic acid alone, and the corresponding oxalic acid alone were always run parallel to the test samples. Excess surface liquid was removed by blotting with cheesecloth.

Video images of the samples were captured in a light box (Luzuriaga and others 1997) immediately after treatment (at 0 h) and at every 1 h interval during storage at room temperature (23 °C) for up to 5-7 h. The samples during the experiment were stored on a plastic screen mesh in a closed container, containing tap water just under the screen, to minimize drying. Four slices were analyzed for each treatment. The experiment was repeated using a different batch of apples and bananas, 2 weeks later.

Color Data Analysis

Browning inhibition was analyzed using a machine-vision system with D65 illumination (Luzuriaga 1999; Chapter 4). Average L* values of the pixels representing the total surface area of each of the apple and banana slices (4 replicates for each treatment level) were analyzed to assess the extent of browning using Color Expert software (Engineering & Cyber Solutions, 2003). L* parameter indicates brightness and darkness from white (100) to black (0). Degree of browning on cut surfaces was evaluated based on experimental parameters (treatment level and type and storage time) using three different approaches:

- Observing changes in $L_0^* - L_t^*$ values, calculated by subtracting the values obtained immediately before storage (L_0^*) from the values obtained after storage (Lt^*).

- Comparing the percent differences (browning index) between the controls and treatments ΔL^* values after a specified storage time t (Sapers and Douglas 1987) as shown in Equation 4-1.

- Comparing temporal changes in color spectra of the samples (controls and treatments) using the Color Expert software (Engineering & Cyber Solutions, 2003) as described previously in Chapter 4. Color was represented in the 512-block RGB system. The R, G, and B axes are divided into 8 regions, each. Any color that falls into one of the resulting 512 blocks is represented by the center color of the block. The average colors of the 4 replicates at each treatment level, corrected by area, were calculated. The color block that changed more than 5% over storage time were selected and compared.

Statistical Analysis

Color data were analyzed in MINITAB statistical software using one-way analysis of variance (ANOVA). ANOVA's were calculated at every time step. Comparison of means was done using the least significance difference (LSD) test.

Determination of Oxalic Acid Concentration in the Treated Tissues

An oxalate kit (Oxalate Urinalysis Diagnostic Kit: Procedure Number 591, Sigma, St. Louis, M.O., U.S.A.) was used to determine the concentration of oxalic acid in apple tissues treated with oxalic acid. The procedure described by Ferrar and Walker (1993) and in Chapter 4 was used for preparation of the samples for oxalic acid analysis. Treated apple tissues, homogenized with an equal weight of 0.2 M KH_2PO_4/Na_2HPO_4, pH 6.0 buffer, respectively, were centrifuged at 10000 x g for 15 min. Fractions (5 mL) of the supernatant were then assayed for oxalic acid.

Results and Discussion

Effect of Ascorbic Acid on Browning Inhibition

The effect of ascorbic acid on browning of sliced apple and banana is shown in Figure 5-1. An observed progressive increase in browning was observed for both

water-dipped controls and the samples treated with 0.5% ascorbic acid, indicating that ascorbic acid alone failed to inhibit browning. These results are not surprising as ascorbic acid alone is generally not a long-lasting antibrowning agent. Typically browning proceeds after the exhaustion of ascorbic acid and the dehydroascorbic acid formed during the oxidation reaction is not involved in browning inhibition (Sapers and others 1989; Mcevily and others 1992; Sapers 1993; Nicolas and others 1994). Ascorbic acid inhibits enzymatic browning by preventing accumulation of *o*-quinones, or it may form stable colorless products (Golan-Goldhirsh and others 1992; Mcevily and others 1992; Sapers 1993; Nicolas and others 1994). Higher concentrations of ascorbic acid were not tested because previous studies by Luo and Barbosa-Canovas (1995) found ascorbic acid to impose an unpleasant sour taste in apple fruit at concentrations above 0.75%.

Antibrowning Potency of Oxalic Acid in Conjunction with Ascorbic Acid on Apple Slices

Changes in browning of apple slices treated in solutions containing 3 different combinations of oxalic acid (1.5, 3 and 5 mM) with ascorbic acid (0.5%) are shown in Figure 5-2. The intensity of browning on the surfaces was evaluated based on the experimental variables (treatment type and level, and storage time) and the reproducibility between the replications in overall trend between the experiments (Figure 5-2). Browning inhibition was brief and a gradual increase in discoloration was observed with treatments containing 1.5 and 3 mM oxalic acid alone during the 7 h storage period. The most effective color preservation among the oxalic acid controls was obtained with treatments containing 5 mM oxalic acid, regardless of the storage period. This observation is consistent with the previous results from Chapter 4. The minimal concentrations of oxalic acid in solutions and in rhubarb juice to effectively suppress

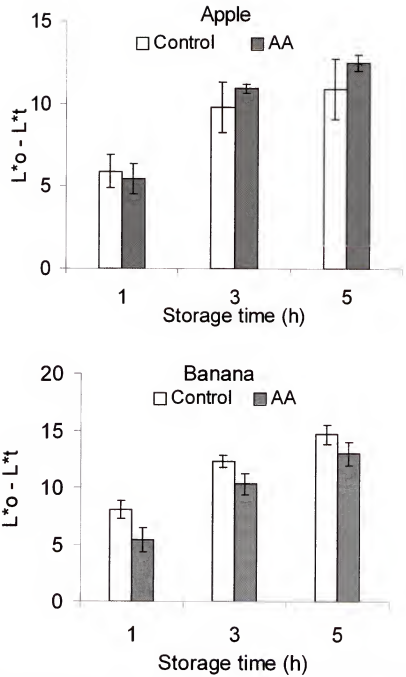


Figure 5-1. Effect of ascorbic acid on browning inhibition of apple and banana slices. Samples were dipped in water (Control) and 0.5% ascorbic acid (AA). Each data point represents mean±standard deviation of 4 replicates

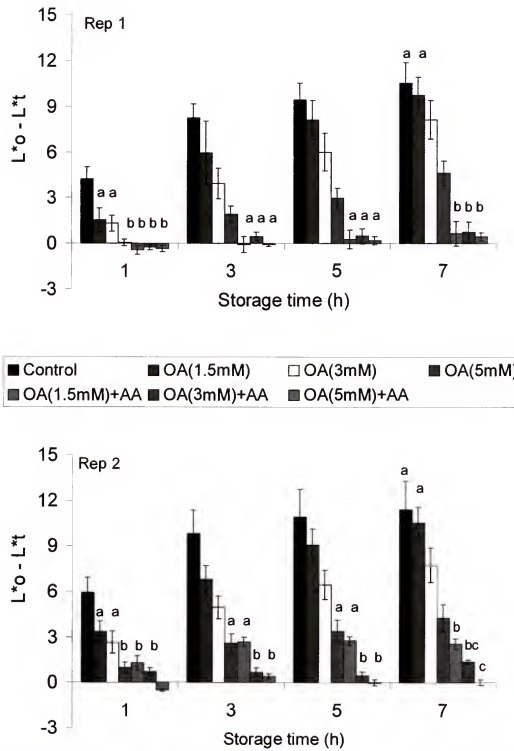


Figure 5-2. Effect of oxalic acid in conjunction with ascorbic acid on browning inhibition of apple slices. Samples were treated with oxalic acid (OA) only and OA plus 0.5% ascorbic acid (AA). LSD tests at $\alpha = 0.05$ were performed for treatments at each interval time and any treatments that were not significantly different from one another are shown as the same letter on the bar graphs

browning of Liberty apples for up to 3 h storage at room temperature were reported to be as low as 0.05 and 0.07%, respectively (Son and others 2000b, 2001).

However, the combination of oxalic acid with ascorbic acid was more effective on browning of the apple slices than ascorbic acid (Figure 5-1) or oxalic acid alone (Figure 5-2). There was no significant difference in relative antibrowning potency of these treatments ($p<0.05$), regardless of the storage period (Figure 5-2, Rep 1). Little variation in the extent of browning inhibition was noticed with the treatments containing 1.5 mM oxalic acid plus ascorbic acid in Rep 2. This latter treatment was still more effective compared to the 5 mM oxalic acid treatment at the 7 h storage. Differing degrees of browning inhibition both between and within cultivars was attributed to apple-to-apple variability (Sapers and others 1989; Sapers and Douglas 1987; Chapter 4). Changes in marketing and environmental conditions during ripening of the fruit may be plausible explanations for the variability in natural pigmentation and texture, as well as the degree of enzymatic browning and its inhibition within cultivars (Barrett and others 1991; Nicolas and others 1994).

Browning indices for oxalic acid-treated apples are shown in Figure 5-3. Degree of browning on treated samples was compared with that of the corresponding control samples on a percent basis (Sapers and Douglas 1987), and is often used to evaluate effectiveness of a browning inhibitor (Sapers and others 1989; Molnar-Perl and Friedman 1990; Lozano-De-Gonzalez and others 1993; Chapter 4). Browning index values approaching 100 indicate that a treatment is highly effective in controlling browning of apple slices (Sapers and Douglas 1987).

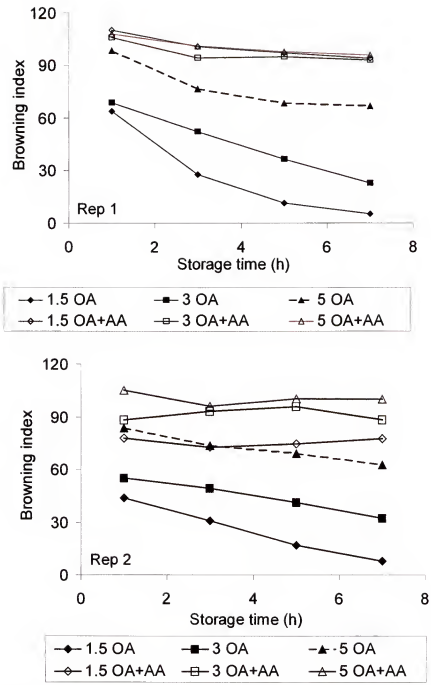


Figure 5-3. Browning indices on cut surfaces of oxalic and ascorbic acids treated apple

There was a positive correlation between browning indices and the level and type of treatments during the storage period (Figure 5-3). An increasing trend in browning index was observed with increasing oxalic acid concentration, indicating the tendency of sliced apple to turn brown decreased with increasing oxalic acid concentration (Chapter 4). However, browning indices for sliced apple obtained with treatments consisting of combinations of oxalic acid with ascorbic acid were noticeably higher compared to those of the corresponding oxalic acid control treatments during the 7 h storage period (Figure 5-3). This observation is consistent with the results presented in Figure 5-2 that combining oxalic acid with ascorbic acid enhanced browning inhibition of apple slices.

The overall results indicated that the interaction between ascorbic and oxalic acid is synergistic; considerable browning occurs when 0.5% ascorbic acid alone (Figure 5-1) and less than 5 mM oxalic acid alone were used (Figures 5-2 and 5-3). The addition of only 0.5% ascorbic acid with the lowest oxalic acid level (1.5 mM) results in an effective browning inhibition up to 7 h (Figures 5-2 and 5-3). Combining ascorbic acid (1%) with oxalic acid (0.02%) extended inhibition for Liberty apples exposed to air for 3 h at room temperature (Son and others 2001). However, a high concentration of ascorbic acid (above 0.75%) was previously demonstrated to impose an unpleasant sour taste in the apple fruit (Luo and Barbosa-Canovas 1995).

The overall results of this study suggested that variation in the effective oxalic and ascorbic acid levels to obtain browning-free apples is expected among apple cultivars because the extent of browning varies among apple cultivars (Coseteng and Lee 1987; Sapers and Douglas 1987). PPO activity and its substrate concentration were reported to

be the two major factors responsible for the differences in degree of browning among apple cultivars (Coseteng and Lee 1987). Red Delicious apple was reported to have the highest PPO activity (Janovitz-Klapp and others 1989) and one of the apple cultivars subjected to severe browning (Sapers and Douglas 1987).

The effect of ascorbic acid, oxalic acid, and combinations of oxalic acid with ascorbic acid on browning inhibition of the apple slices was also analyzed for temporal color changes using the Color Expert software (Engineering & Cyber Solutions, 2003). The results are presented in Figure 5-4. Color spectra of the water-dipped samples (control) and ascorbic acid treated samples drastically changed during the storage period (Figure 5-4). A sharp decrease in the level of lighter colors (color blocks 501 to 509) followed formation of darker colors (color blocks 500 and greater) after the 3 h storage period for both water-dipped controls and ascorbic acid treated samples. However, a gradual decrease in the level of the lighter colors was observed for the sliced apples treated with 1.5 and 3 mM oxalic acid solutions compared with the control samples (Figure 5-4). The color spectra of these latter treatments and the controls shifted from the lighter colors to the darker colors. There was practically no change in the color spectra of the sliced apples treated with 5 mM oxalic acid solutions. This observation was consistent with the results from Chapter 4 where oxalic acid was shown to effectively control color deterioration of sliced apples at a concentration of 5 mM or higher. However, the most effective color preservation was obtained with treatments consisting of combinations of oxalic acid (1.5, 3, and 5 mM) and ascorbic acid (0.5%) (Figure 5-4). The color spectra for the sliced apples treated by any of these combinations was comparable, regardless of the storage period.

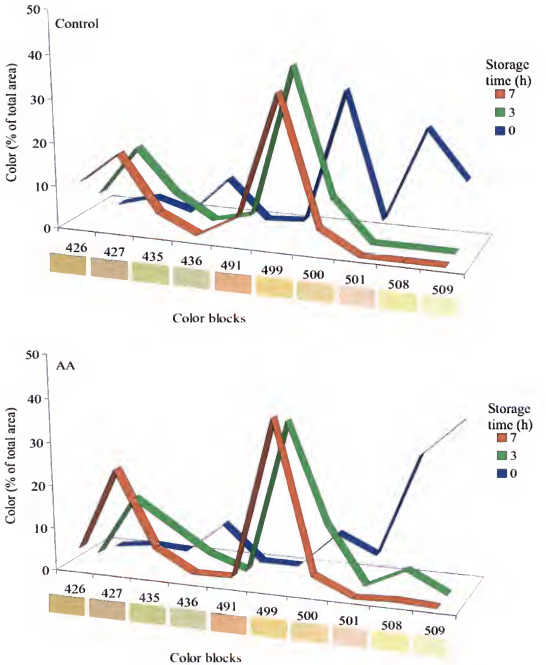


Figure 5-4. Temporal changes in color spectra on cut surfaces of oxalic acid and ascorbic acid treated apple. Control samples were dipped in water. Color was represented in the 512-block RGB system

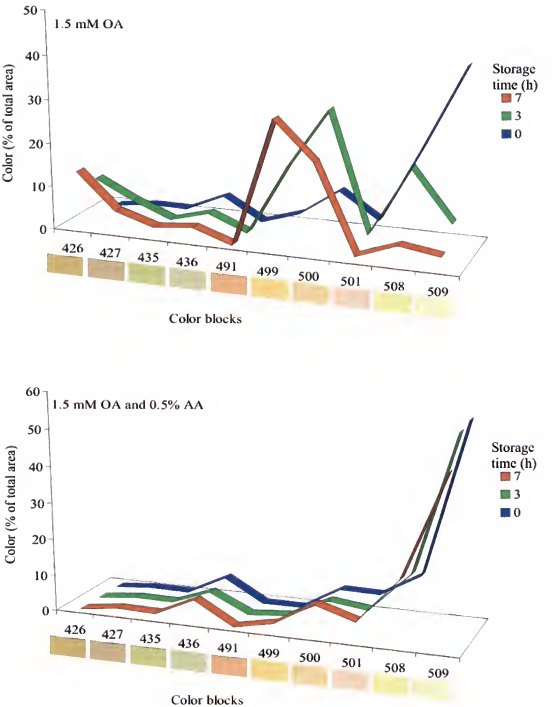


Figure 5-4 Continued

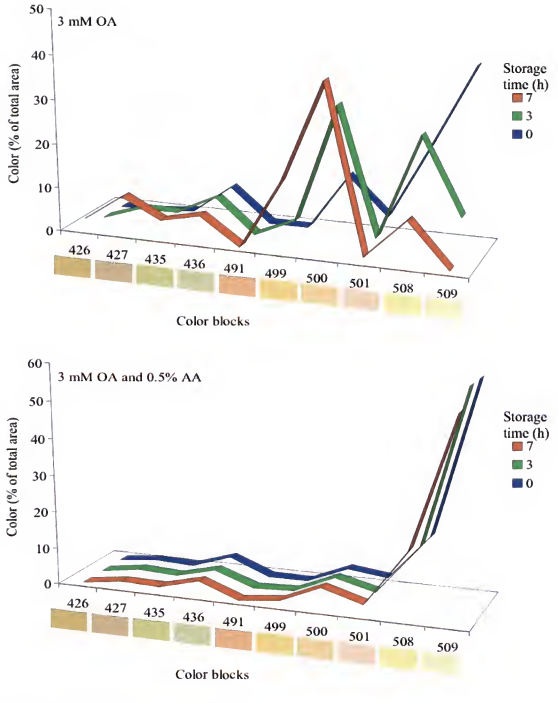


Figure 5-4 Continued

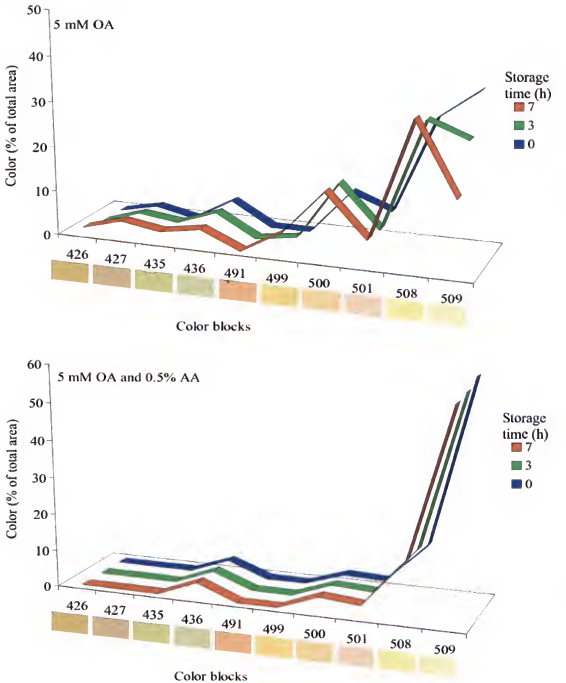


Figure 5-4 Continued

The level of the lighter colors on the surfaces treated in combinations of oxalic acid with ascorbic acid was slightly higher compared to those treated in oxalic acid alone (Figure 5-4). This might be due to the higher effect of browning inhibition of the former treatments during the dipping period and there might be some pigment bleaching as well. Previous studies in our laboratory demonstrated that incubation of oxalic acid (10 mM) with the final product(s) of the PPO-catechol reaction has no influence on the level of the pigmented oxidation products (Chapter 3). However, ascorbic acid changed the absorption spectra by reducing the quinones back to the original colorless diphenol, which delayed the formation of melanin (Chapter 3).

The mechanism for improvement of browning inhibition on sliced apples by 4-hexylresorcinol and ascorbic acid was attributed to the inhibition of apple PPO activity by 4-hexylresorcinol, and thereby, prolonged retention of ascorbic acid (Luo and Barbosa-Canovas 1995). A similar assumption can be drawn to explain the mechanism of browning inhibition achieved with oxalic acid in conjunction with ascorbic acid on sliced apples (Figures 5-2, 5-3, and 5-4).). Inhibition of PPO by oxalic acid is very likely due to its binding, chelation, and removal of the active-site copper from the enzyme (Sato 1980b; Son and others 2000a; Chapter 3). Hence, oxalic acid is similar to EDTA (ethylenediaminetetraacetic acid) in its mode of action, and might also act to stabilize ascorbic acid by inhibiting the metal catalyzed oxidation of ascorbic acid through its chelating action (Furia 1964). Moreover, the stability of ascorbic acid in an acidic environment and the inhibitory effect of the acidic environment on the catalytic activity of the enzyme might be additional factors that explain browning inhibition on sliced apples using oxalic acid in conjunction with ascorbic acid.

Oxalic acid was shown to effectively suppress browning of Red Delicious apples in this study (Figure 5-2, 5-3, and 5-4) and of Liberty apples by Son and others (2001) at concentrations well below the level found naturally in the foods presented in Table 2-2. Tissue concentrations of oxalic acid dihydrate, at effective antibrowning activity in sliced Red Delicious apples treated with 3 and 5 mM oxalic acid solutions, were 5.8 ± 0.05 and 9.8 ± 0.06 mg/100 g fresh weight, respectively. Oxalic acid suppressed browning of Liberty apples for 3 h at low concentrations (1.6-2.5 mg/100 g fresh material), where the apple slices were treated with 0.05% oxalic acid solution (Son and others 2001). Even though there does appear to be a prospect of using oxalic acid at low levels in conjunction with ascorbic acid to control browning of minimally processed apples, further studies are required to address this issue from a nutritional point of view.

Oxalic acid in both soluble and insoluble forms is a natural component of many higher plants (Table 2-2) and it was suggested to have some selective, functional significance or adaptation during normal plant development (Ilarslan and others 1997). Knowledge of oxalic acid concentration in food products is important, as oxalic acid at high levels in the diet tends to reduce magnesium and calcium availability in humans (Prenen and others 1984; USDA 1984). However, it was reported that the oxalic acid rich foods usually containing sufficient calcium or magnesium would not interfere with the calcium and magnesium availability of other foods in the diet (USDA 1984). The daily consumption of oxalic acid in Western diet was reported to be within the range of 70 to 150 mg (Son and others 2001).

Antibrowning Potency of Oxalic Acid in Conjunction with Ascorbic Acid on Banana Slices

Changes in browning of banana slices treated in solutions containing oxalic acid only and oxalic acid plus ascorbic acid are shown in Figure 5-5. The 10 and 20 mM oxalic acid solutions showed similar effectiveness in browning inhibition ($p<0.05$). Dipping the slices in solutions containing 40 mM oxalic acid significantly reduced the browning of bananas ($p<0.05$) during 5 h storage period. However, gradual discoloration was noticed in the center of the fruit slices. Similar observations were reported previously in Chapter 4 where we observed the best color preservation with solutions containing as low as 60 mM oxalic acid with relatively less discoloration in the center of the fruit. The intense browning in the center of banana fruit is related to the localization of PPO mainly in this area (Galeazzi and others 1981).

Combining 3 different combinations of oxalic acid (10, 20 and 40 mM) with ascorbic acid (0.5%) did not improve browning inhibition for banana slices (Figure 5-5). Browning inhibition accomplished with any of these oxalic acid plus ascorbic treatments was not significantly different from the corresponding oxalic acid controls ($p<0.05$), regardless of the storage period. Browning indices (Figure 5-6) that measure the degree of browning of the treated samples relative to the water-dipped controls were consistent with the results presented in Figure 5-5. The browning index profiles of the treatments and the corresponding oxalic acid controls were comparable. Dipping banana slices in 60 mM oxalic acid solutions was highly effective in controlling browning of banana slices, and generated an average residue of 85 mg/100 g fresh weight (Chapter 4). Although this level of oxalic acid is less than levels naturally occurring in such oxalic acid rich foods as spinach, Brussels sprouts, etc. (Table 2-2), it appears that the prospect of using oxalic

acid alone or in combination with ascorbic as an antibrowning agent on banana may have limitations because of nutritional implications of metal binding (Chapter 3 and 4). Hence, additional nutritional studies are needed to address this issue.

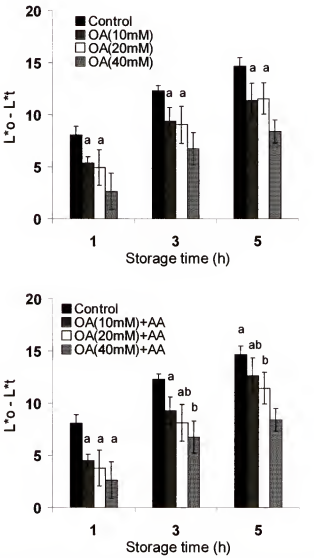


Figure 5-5. Effect of oxalic acid in conjunction with ascorbic acid on browning inhibition of banana slices. Samples were treated with oxalic acid (OA) only and OA plus 0.5% ascorbic acid (AA). LSD tests at $\alpha = 0.05$ were performed for treatments at each interval time and any treatments that were not significantly different from one another are shown as the same letter on the bar graphs. The experiments were repeated using a different batch of banana 2 weeks later and the reproducibility between the replications in overall trend between the experiments were similar (data not shown)

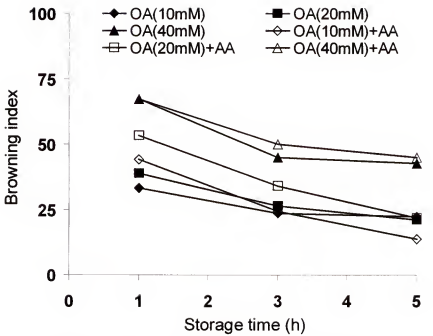


Figure 5-6. Browning indices on cut surfaces of oxalic acid and ascorbic acid treated banana

The performance of oxalic acid as a browning inhibitor appears to be dependent on the specific food system. In a previous study, inhibitory activity of 3.3 mM oxalic acid tested on banana and apple PPOs in a reaction model system was found to be 21 and 100%, respectively (Ferrar and Walker 1996). Hence, the plausible explanation for the striking difference in inhibitor potency of oxalic acid and oxalic acid plus ascorbic acid on the browning of apple (Figure 5-2, 5-3, 5-4) and banana slices (Figure 5-5, 5-6) might be the differences in physiochemical properties of PPOs. The enzyme has been isolated and characterized from numerous fruits and vegetables and its physiochemical properties, such as substrate specificity, pH and temperature optima, molecular weight, multiple forms and sensitivity to inhibitors, show considerable variations among sources (Vamos-Vigyazo 1981; Yoruk and Marshall 2003). Therefore, the success of inhibition may vary with the type and concentration of inhibitor, and PPO source, and specific

control measures for the individual systems would be needed (Sapers and others 1989; McEvily and others 1992; Ferrar and Walker 1996). Additionally, differences in the level of PPO activity and natural pigmentation between apple and banana fruits might also account for variability in the extent of browning inhibition by oxalic acid. However, additional factors, both enzymatic and nonenzymatic, contributing to browning in individual commodities can not be overlooked to better control browning of minimally processed fruits and vegetables (Vamos-Vigyazo 1981; Sapers 1993; Nicolas and others 1994; Yoruk and Marshall 2003).

CHAPTER 6
APPLE POLYPHENOL OXIDASE INHIBITOR(S) FROM COMMON HOUSE FLY (*Musca domestica* L.)

Introduction

Enzymatic browning, mainly associated with polyphenol oxidase (PPO; EC 1.10.3.1), causes millions of dollars in damage yearly to fruits, vegetables, and seafood (crustaceans) (Whitaker and Lee 1995; Kim and others 2000). PPO catalyzes the hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols into *o*-quinones (diphenolase activity) with oxygen as the primary oxidant (Whitaker 1994; Lerch 1995; Yoruk and Marshall 2003). The highly reactive quinones usually undergo further nonenzymatic condensation reactions leading to the generation of unpleasant dark brown melanins (Mathew and Parpia 1971; Sapers 1993). Quality of many fruit and vegetable crops susceptible to this biochemical reaction diminishes rapidly due to unpleasant discoloration and off-flavor generation (Vamos-Vigyazo 1981; Zawistowski and others 1991; Yoruk and Marshall 2003).

In most cases, catalytic action of PPO is a limiting factor in the handling and processing of fruits and vegetables since peeled, sliced, bruised, or diseased tissues rapidly undergo adverse enzymatic browning. Severe browning of plant products arises from stress conditions due to subcellular decompartmentelization and oxygen penetration leading to PPO-substrate contact (Vamos-Vigyazo 1981; Zawistowski and others 1991). Many proposed methods for controlling PPO are not applicable to foodstuffs because of problems related to food safety, regulations, and the cost associated with prevention

(Mcevily and others 1992; Sapers 1993). One group of preservatives, sulfites and related substances, are very effective in preventing browning; however, they may cause harm to individuals who are asthmatic. Therefore, their use has been limited on fresh fruits and vegetables (Sapers 1993). There is interest in effective, natural, and inexpensive alternatives.

In addition to its general occurrence in plants, PPO is found in insects (Brunet 1980; Sugumaran 1998). During insect development, PPO is believed to be responsible for various physiological roles. It is involved in sclerotization (hardening) and tanning of the insect cuticle (Sugumaran 1998) and in defense reactions, wound healing, and disease resistance (Gillespie and others 1997; Sugumaran and Nellaiappan 2000). It stands to reason that natural inhibitors within the house fly regulate this development and previous literature has indicated endogenous inhibitors to house fly PPO (Tsukamoto and others 1992; Daquinag and others 1995, 1999). Additionally, Sugumaran and Nellaiappan (2000) have isolated a high-molecular-weight (380000) glycoprotein inhibitor of PPO from the larval cuticle of the tobacco horn worm, *Manduca sexta*, and proposed that the presence of endogenous PPO inhibitor(s) would ensure the direct control of PPO activity in insects.

The potent PPO inhibitors isolated from the pupae of house fly on house fly PPO were found to be heat-stable low-molecular-weight (approximately 3000 to 4200) peptides with an inhibition constant in the nM range (Tsukamoto and others 1992; Daquinag and others 1995). There is little, if any, documentation on the potential applicability of these inhibitors to food systems. The objectives of this study were to provide evidence for the existence of apple PPO inhibitor(s) in common house fly and to

demonstrate inhibition of apple PPO activity using inhibitor(s) isolated from house fly pupae. PPO inhibition profile of the house fly during its growth and development was also evaluated.

Materials and Methods

House Fly Pupae

Pupae of *Musca domestica* (L.) were provided by the U.S. Dept. of Agriculture, Center for Medical, Agricultural, and Veterinary Entomology (Gainesville, FL). Larvae of the house fly were reared on the Gainesville House fly Diet (Hogsette 1992). The diets seeded with house fly eggs were held at 26.7 °C and 60% relative humidity (RH). After 6 days, pupae were separated from the culture medium by flotation. They were further washed in distilled water, and dried on a paper towel. Care was taken to be sure that pupae samples were free of the culture medium. Figure 6-1 illustrates some laboratory materials used for production of house fly pupae.

Preparation of Inhibitor Extract

Pupae obtained 2 days after pupation (middle stage pupae) were powdered in a Waring blender after freezing in liquid nitrogen and stored at –20 °C until used for inhibitor extraction. The powder, homogenized in 4-fold (w/v) 0.1 M sodium acetate-acetic acid, pH 4.0 buffer, was filtered through 8 layers of cheesecloth and then incubated in a water bath at 70 °C for 30 min (Tsukamoto and others 1992). The resulting extract was centrifuged at 4 °C and 30000 x g for 30 min to remove coagulated materials. The supernatant, dialyzed in a 2000-Da molecular-weight cut-off (MWCO) dialysis membrane (Spectrum Laboratory Products, Inc., New Brunswick, NJ) with constant stirring for 25 h against 3 changes of distilled water, was tested for PPO inhibition and properties of inhibitor(s). Dialysates were kept at 4 °C.

Larval rearing trays

Developing larvae in the Gainesville House fly diet

House fly adult cages

Inside a cage showing water, food, and egg collector

Figure 6-1. Pictures of some laboratory materials for production of house fly

In the experiments designed to study the inhibitor activity of house fly during growth and development, samples from the following stages were collected: 3rd-instar larvae, 5 different pupae samples obtained throughout pupation, and adults. Larvae were removed from the culture medium by using Berlese funnel as illustrated in Figure 6-2. House fly samples were stored at –20 °C until needed. They were directly used for extraction. Crude extracts prepared from samples (10 g) at each stage of the development by buffer extraction and heat treatment, as described above, were tested for PPO inhibition activity. The experiments were repeated twice each with 3 replicates.

Preparation of Apple PPO

Red Delicious apple was chosen as a PPO source because it was previously shown to have the highest enzyme activity among many apple cultivars (Janovitz-Klapp and others 1989). Apple PPO was extracted from an apple acetone powder. Acetone powder was prepared from Red Delicious apples obtained from a local grocery store as described in Chapter 3. The white apple powder was vacuum-dried at room temperature (25 °C) and stored in commercial vacuum bags at –20 °C until used for PPO extraction.

The procedure described in Chapter 3 was used for PPO extraction. The acetone powder (1 g) was incubated in 50 mL of 0.1 M KH_2PO_4/Na_2HPO_4, pH 7.2 buffer containing 1% Triton X-100 (Bio-Rad Laboratories, Hercules, CA) for 20 min while stirring with a magnetic stirrer at 4 °C. The suspension was centrifuged for 30 min at 12000 x g, and the resulting supernatant, filtered through glass wool, was tested for PPO activity and properties of inhibitor(s). PPO activity of apple acetone extract kept in the freezer (-20 °C) remained stable for a week with no visible intrinsic phenolic oxidation.

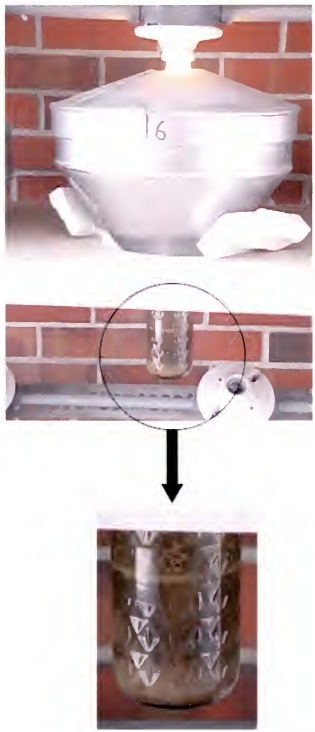


Figure 6-2. Autoseparation of house fly larvae from culture medium using Berlese funnel

Assay of PPO Activity and Inhibition

PPO inhibition was quantified by measuring PPO activity of apple with and without added inhibitor extract. For a standard assay, 0.2 mL of test extract or control buffer was mixed with 2.45 mL of 0.1 M sodium acetate-acetic acid, pH 5.5 buffer as the main buffer, 0.3 mL of 0.5 M catechol (Sigma Chemical Co., St. Louis, MO) and 0.05 mL of the enzyme extract. PPO activity was measured by spectrophotometric determination of initial reaction rates at 420 nm and 25 °C with a Beckman Model DU 640 Ultraviolet-Visible spectrophotometer. The pH of the reaction mixture was checked after each assay to confirm similar pH values for control and test systems.

PPO activity of apple also was assayed by mixing it with an inhibitor extract in a standard reaction mixture where the pH of the main buffer (0.1 M sodium acetate-acetic acid) varied from pH 4.0 to 5.7. One unit of enzyme activity was defined as an increase in absorbance of 0.001 per min at 25 °C. The degree of inhibition on the reaction was expressed as percent inhibition (I), calculated using the formula [100(A-B)/A], where A and B were enzyme activities in the control and test systems, respectively. Specific activity of inhibition for house fly stages was expressed as unit (U) inhibition per mg protein, where 1 U of inhibitor activity was defined as 10% inhibition of apple PPO.

The pH Optimum of Apple PPO

The pH-activity profile for the oxidation of catechol by PPO was determined using 0.1 M sodium acetate, pH 4.0 to 5.5, and 0.1 M sodium phosphate, pH 6.0 to 7.0. The reaction mixture contained 0.1 mL of the enzyme solution, 0.3 mL of 0.5 M catechol and 2.6 mL of various buffer solutions. The reaction was initiated with the addition of the substrate prepared in distilled water. PPO activity was estimated as described above.

Effect of Heating and Freezing and Thawing on Inhibitor(s)

Aliquots of 2 mL of the inhibitor preparation were incubated in boiling water, 100 °C, for 15, 30, and 60 min. The precipitate was separated by centrifugation for 5 min at 10000 x g and room temperature (22 °C). The clear supernatants were collected in clean test tubes and used for PPO inhibitory activity determination at 25 °C as described above.

Repeated freezing and thawing (5 times) on crude inhibitor preparation also was determined. In the last freezing step, the samples were thawed and assayed after storing them overnight at –20 °C.

The pH Stability of Inhibitor(s)

A crude inhibitor extract (prior to dialysis) adjusted to pH values from 3.0 to 11.0 with HCl or NaOH was incubated for 5 h at the adjusted pH at refrigeration temperature (4 °C). Volume differences between samples were adjusted with distilled water and inhibitor samples were dialyzed using a 2000-Da MWCO membrane for 30 h against 3 changes of distilled water. Dialysates were centrifuged at room temperature for 5 min. Supernatants were tested for PPO inhibition as described above.

Gamma Irradiation

Since house fly pupae are routinely irradiated for preservation purposes, the pupae were exposed to 500 Gray (Gy) of gamma irradiation in a cesium-137 irradiator at the USDA laboratory in Gainesville, FL, according to Morgan and others (1986). The irradiated pupae, subjected to buffer extraction and heat treatment as described above, were used as crude inhibitor extract. PPO activity of apple mixed with inhibitor extract in a standard reaction mixture was measured spectrophotometrically as described above.

Ultrafiltration

Ultrafree-4 centrifugal filter units (Spectrum Laboratory Products, Inc.) with the nominal molecular weight limit (NMWL) membranes of 10000, 30000, 50000, and 100000 were used. After rinsing with distilled water to remove a trace amount of glycerin, aliquots of 2 mL of the inhibitor sample were placed onto the filter units and concentrated by centrifugation at 4 °C and 7500 x g for 35 min. Filtrates were collected and kept at 4 °C. The sample was reconstituted back to 2 mL by adding distilled water and centrifuged again. Two wash cycles were performed. Concentrated inhibitor sample on the membrane surface (retentate) was reconstituted back to 2 mL by adding distilled water and assayed for PPO inhibitory activity as described above. Control inhibitor samples in centrifuge tubes also were subject to centrifugation with test samples together.

Protein Determination

Protein was determined using bovine serum albumin (Sigma Chemical Co.) as a standard (Bradford 1976).

Preliminary Purification Studies

Acetone precipitation

The pupal inhibitor extract prepared by buffer extraction and heat treatment at 70 °C for 30 min (described above) was subjected to acetone precipitation by using the method described by Jayaraman and others (1987) with some modifications. The precipitate obtained by slow addition of 1 volume of cold acetone (Fisher Scientific, Fair Lawn, NJ) at –25 °C was collected by centrifugation at 20000 x g for 20 min at 0 °C. The resulting pellet was suspended in a minimal volume of 50 mM sodium phosphate buffer (pH 6.0) and dialyzed using a 3500-Da MWCO membrane (Pierce Biotechnology, Inc,

Rockford, IL) overnight against the same buffer. A similar fractionation method also was performed using cold butanol.

Ammonium sulfate precipitation and column chromatography

Another attempt to precipitate the house fly inhibitor(s) was ammonium sulfate saturation. The pupal inhibitor extract was brought to 95% saturation using solid ammonium sulfate (Fisher Scientific) with continuous stirring at 0°C. After incubation overnight in the cold room (4 °C), the solution was centrifuged at 10000 x g for 30 min at 4 °C. The precipitate was dissolved in a minimal volume of 50 mM sodium phosphate buffer (pH 6.0). The salt was removed by dialysis in the same buffer for 40 h with 3 changes of buffer at 4 °C.

The dialyzed inhibitor extract was clarified by centrifugation at 30000 x g for 30 min, then applied to a Sephadex G25-column (2.5 x 48 cm, Sigma Co.) previously equilibrated with 50 mM sodium phosphate buffer (pH 6.0). The void volume of the column was determined using blue dextran (Sigma Co.). The eluted protein was monitored by measuring the absorbance at 280 nm. PPO inhibition was measured by spectrophotometric determination of initial reaction rates at 420 nm as described above. Active fractions were pooled and chromatographed in a Toyopeal 550C column (2.5 x 28 cm, Sigma Co.) prequilibrated with 50 mM sodium phosphate buffer (pH 6.0). The column was eluted with the equilibration buffer; and the eluted protein was monitored by measuring the absorbance at 280 nm. After the absorbance had returned to the baseline, further elution was carried out with a linear salt gradient from 0 to 1 M NaCl in 50 mM sodium phosphate buffer (pH 6.0). The absorbance at 280 nm and PPO inhibition were determined in each 6-mL fraction.

Results and Discussion

Optimization of pH Conditions for PPO Activity and Inhibition

The pH activity profile for oxidation of catechol by apple PPO is shown in Figure 6-3. PPO showed maximum activity at pH around 5.5. About 68% activity, relative to that determined at pH 5.5, was detected at pH 5.0, but there was barely any activity at or above pH 6.0. The acidic pH optimum for apple PPO was also detected by other researchers but some found a single pH optimum at pH 4.5 to 5.0 (Janovitz-Klapp and others 1989; Zhou and others 1993), whereas others noted the existence of two pH optima, one at around pH 7.0 and one at around pH 5.0 (Stelzig and others 1972). Experimental factors such as extraction methods, temperature, nature of the phenolic substrate, and buffer system used during determination and source differences can affect the optimum pH value for PPO (Stelzig and others 1972; Janovitz-Klapp and others 1989; Zhou and others 1993; Whitaker 1994).

The presence of inhibitor(s) in house fly pupae was demonstrated spectrophotometrically by mixing the pupal extract prepared by buffer extraction, heat treatment, and dialysis with an active preparation of apple PPO at a reaction pH optimal for the enzyme activity (pH 5.5). Decreased enzyme activity relative to the control is shown in Figure 6-4. The crude inhibitor preparation reduced apple PPO activity by 80%. Inhibition was rapid, occurring in the reaction mixture, and did not require incubation. Further studies of PPO inhibitor(s) assay conditions revealed the importance of reaction pH on inhibition activity by the pupal extract (Figure 6-5). Inhibition by the fly extract on apple PPO markedly differed as a function of pH. There was barely any inhibition at or below pH 5.0. Above pH 5.0, inhibition showed an increasing trend with increasing pH values tested. Greatest inhibition (90%) of PPO was observed at pH 5.7, however, this

increased inhibition may occur as a result of the decline in PPO activity without inhibitor(s) as a function of pH (Figures 6-3 and 6-5). It should also be noted that under the optimal pH conditions (pH 5.5) for PPO activity, the house fly inhibitor(s) demonstrated only a 10% reduction in inhibition when compared with pH 5.7.

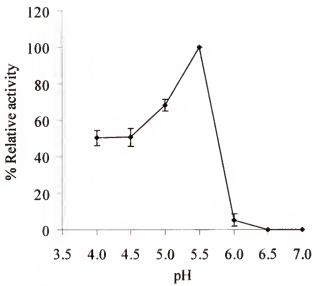


Figure 6-3. The pH optimum of apple PPO. Activities are expressed as % relative activity from the activity determined at pH 5.5. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates

Tsukamoto and others (1992) isolated inhibitors from the pupal extracts of houseflies and demonstrated inhibition on house fly PPO. The presence of endogenous inhibitor in the larval cuticle of another insect, the tobacco horn worm (Manduca sexta), was reported by Sugumaran and Nellaiappan (2000). The specific endogenous PPO inhibitor of Manduca sexta inhibited the activity of not only insect PPOs but also plant PPOs and laccase. In addition to several other regulatory biochemical mechanisms of endogenous PPO activity in insects (Sugumaran and Nellaiappan 2000), it is believed that PPO activity could also be regulated by intrinsic inhibitors during development (Tsukamoto and others 1992; Daquinag and others 1995, 1999; Sugumaran and Nellaiappan 2000).

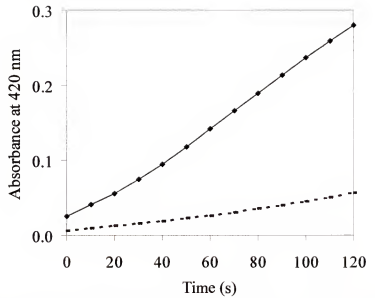


Figure 6-4. Inhibition of apple PPO activity by inhibitor(s) from house fly pupae. PPO alone (—); PPO plus inhibitor(s) (----). Inhibition activity was observed at 48 μg house fly protein in the 3-mL standard reaction mixture.

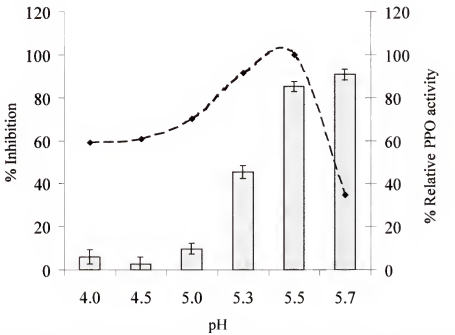


Figure 6-5. Optimum pH for PPO inhibition by pupal extract. This was determined by measuring the PPO activity of apple mixed with inhibitor(s) (48 μg protein) in a standard reaction mixture where the pH of the main buffer (0.1 M acetate-acetic acid) varied from 4.0 to 5.7. Bars indicate % inhibition while the dashed line shows % relative PPO activity without inhibitor(s). Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates

Inhibitor Activity of the House Fly During Metamorphosis

Figure 6-6 illustrates the developmental stages of house fly (*Musca domestica* L.). The house fly life cycle varies greatly in length depending on environmental factors, such as temperature, moisture level, and the pH of the larval medium (West 1951). The following times were observed through the developmental stages of the laboratory house fly: eggs hatched in 24 h, and the periods for the 3 larval stages or instars and the pupal stage averaged 3 and 5 d, respectively.

PPO inhibition activity in house fly during its growth and development is shown in Figure 6-7. The percent inhibition per 0.2 mL of inhibitor extracts obtained from larvae through adult stages was not much different, especially at pupal stages. The late pupal (P5), larval, and adult stages showed slightly lower percent inhibition. However, specific inhibitor(s) activity (U inhibition / mg protein) showed an influence in inhibition throughout the developmental stages, where highest inhibition was observed in the larvae stage. Based on specific activity of inhibitor(s), apple PPO inhibition was relatively lower with the inhibitor(s) in early pupae stages (P1 and P2) than middle-stage and late-stage pupae (Figure 6-7). These are contradictory to the findings of Tsukamoto and others (1992) who observed significant variation in the inhibitor activity of the developmental stages (per gram body weight) on house fly PPO, and house fly PPO inhibition was prominent with the inhibitors in aged pupae and little with those in larvae and adults.

In this investigation, none of these house fly extracts exhibited PPO activity under the standard assay conditions containing catechol as substrate. This observation is consistent with the results from Tsukamoto and others (1992) obtained for the inhibitors on house fly PPO with DOPA as substrate.

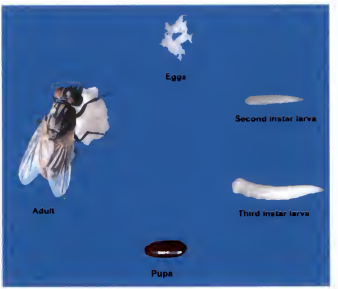


Figure 6-6. Life cycle of house fly. House fly development through the stages of egg, larva, pupa, and adult

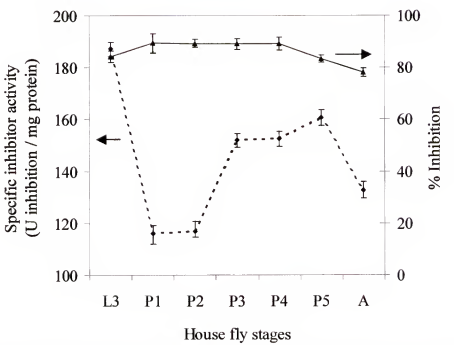


Figure 6-7. Inhibition by house fly extract on apple PPO through its developmental stages. Crude extracts prepared from 3rd instar larvae (L3), young pupae just formed (P1), pupae samples obtained on day 2 (P2), 3 (P3), 4 (P4), and 5 (P5) after pupation, and adults (A) were tested for inhibitory effect on apple PPO in a standard reaction mixture. % Inhibition (—); specific inhibitor activity (----). One unit (U) of inhibitor activity was defined as 10% inhibition of apple PPO. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates

Characterization of Crude Inhibitor(s) From House Fly Pupae

Effect of inhibitor concentration

Crude inhibitor(s) preparation was initially analyzed for its concentration dependency on PPO activity. Serial dilution of the inhibitor preparation demonstrated a decrease in percent inhibition with increasing dilution factor. The overall dilution profile gave an almost logarithmic relationship between inhibitor(s) concentration and activity. However, a linear relationship (r=0.99) was observed upon dilution of the inhibitor between 1/2 and 1/16. About 40% of the inhibitor activity was observed upon dilution by 1/4. Inhibitor activity calculated on the basis of the protein amount found in the standard reaction mixture is shown in Figure 6-8. In this case, about 40% of the activity was observed at 13 μg protein in the 3-mL standard reaction mixture. Higher protein concentration (≥25 μg) in the standard reaction mixture did not cause a substantial increase in inhibition, indicating maximum inhibition was achieved.

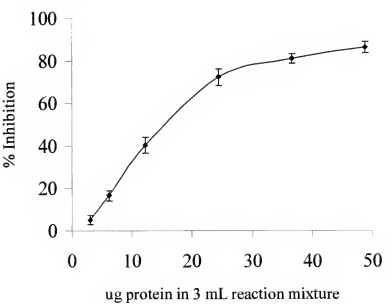


Figure 6-8. Effect of inhibitor concentration on PPO activity. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates

Temperature stability

The effect of heat treatment on the activity of crude inhibitor(s) is shown in Table 6-1. The unknown inhibitor(s) was relatively stable to heat, losing about 26% of its inhibitory activity on apple PPO upon boiling at 100 °C up to 1 h. When relative inhibition was calculated based on specific activity of inhibitor(s) (U inhibition / mg protein), inhibitor(s) retained about 88% of original activity after 1 h of heating. In this respect, inhibitor(s) was similar to those isolated by Tsukamoto and others (1992) who noted that the inhibitors in house fly pupae for house fly PPO are quite stable to heating at 80 °C for 1 h as they retained greater than 60% of their activity. However, the inhibitor isolated from tobacco horn worm was a heat-labile glycoprotein, totally inactivated by heating at 100 °C for 10 min (Sugumaran and Nellaiappan 2000).

Further investigations of the inhibitor(s) from house fly pupae demonstrated that the inhibitor(s) was also stable to repeated freezing and thawing, losing only about 8% of its inhibitor activity. This result indicated that inhibitor(s) are quite stable to freeze-thaw cycle. Inhibitor activity of pupal extract kept at refrigerated temperature (4 °C) remained stable over a period of a week.

Table 6-1. Heating effect on inhibitor activity

	Relative % inhibitor activity	
Heating period (min at 100 °C)	% Inhibition	Specific inhibitor activity (U inhibition/mg protein)[a]
0	100	100
15	86.2 ± 7.9	94.3 ± 9.5
30	77.6 ± 6.0	88.6 ± 8.3
60	73.9 ± 5.2	88.5 ± 6.9

[a] One unit (U) of inhibitor activity was defined as 10% inhibition of apple PPO

The pH stability

Inhibitor(s) exposed to a range of pH values from 3.0 to 11.0 for 5 h was compared for its inhibitory activity on apple PPO. The pH stability curve is shown in Figure 6-9. pH had a dramatic effect on the inhibitor(s). It was most stable at acidic pH values, specifically pH 5.0, and least stable at alkaline pH values. The pH stability of the inhibitor(s) increased from pH 3.0 to 5.0 and showed a rapidly decreasing trend above pH 5.0. The observed inhibitions upon exposure to pH 7.0, 9.0, and 11.0 were about 57, 36, and 38% when compared with those at pH 5.0, respectively (Figure 6-9). However, exposure to pH 3.0 caused only 14% loss in inhibition.

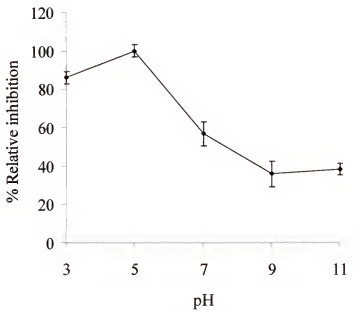


Figure 6-9. The pH stability of inhibitor(s). Inhibitor extract incubated for 5 h at the adjusted pH and 4 °C was dialyzed against de-ionized water and then tested for PPO inhibition. Inhibition activities are expressed as % of that determined at pH 5.0. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates

Sugumaran and Nellaiappan (2000) also reported the dramatic effect of pH on the inhibitor from tobacco horn worm. However, in their case, the inhibitor was most stable

at neutral pH values, losing its total activity upon exposure to pH 10 for 10 min. The findings in our investigation were contradictory to those reported by Tsukamoto and others (1992) who observed that the inhibitor(s) in house fly pupae are quite stable over a wide range of pH (4.0 to 10.0). However, the extent of stability obtained for these particular studies was not presented in their publication.

Gamma irradiation

House fly pupae exposed to irradiation based on a technique using 500-Gy gamma irradiation (Morgan and others 1986) were tested for PPO inhibitory activity to determine whether irradiation influenced inhibition. Based on the results obtained from the investigation, exposing the pupae to 500-Gy gamma irradiation did not cause any change in PPO inhibition effect of the pupal extract. This irradiation technique developed by Morgan and others (1986) was routinely used at the mass-rearing laboratory of the USDA in Gainesville, FL, to supplement the daily production of house fly pupae for maintenance of the parasitoid colonies. The ionizing radiation was shown to prolong the 2- and 3-day-old house fly pupal period from only 24 h to up to 8 wk of storage at around 4.4 °C (Morgan and others 1986). Studies on Caribbean fruit fly larvae exposed to gamma rays revealed reductions in brain growth (Nation and others 1995). Additionally, radiation treatment caused a stress condition in mango fruit, leading to a gross distortion of normal biochemical patterns during ripening and senescence (Frylinck and others 1987).

Ultrafiltration

Inhibitor preparation was further evaluated by ultrafiltration studies and the results are shown in Table 6-2. Because the inhibitor preparation used in these studies was subjected to dialysis in a 2000-NMWL dialysis membrane after heat treatment as

described in Materials and Methods, relative inhibitor activity in the filtrated samples were compared to that in the dialysate (Table 6-2). Ultrafiltration studies were carried out to determine the approximate molecular weight of unknown inhibitor(s) required for future dialysis information during purification and to determine whether it was similar to the peptides referred to by Tsukamoto and others (1992). The overall results showed that there was a decreasing trend in inhibitor activity in the retentate with increasing NMWL of the membranes (Table 6-2). In all cases, the inhibitor activity lost in the retentate was recovered in the filtrate. Ultrafiltration units with 10000, 30000, and 50000 NMWL membranes would retain about 83, 67, and 51% of the inhibitor activity, respectively. However, only 10% inhibition was retained for the 100000 NMWL membranes. It seems that the unknown inhibitor(s) could be larger than 3000 to 3500 found by Tsukamoto and others (1992) based on Sephadex G-25 gel filtration information.

Table 6-2. Ultrafiltration of inhibitor preparation[a]

	Relative % inhibitor activity	
NMWL	Retentate	Filtrate
2000[b]	100	—
10000	83.2 ± 3.1	19.8 ± 4.2
30000	66.5 ± 4.0	35.2 ± 5.6
50000	50.9 ± 6.9	53.7 ± 7.6
100000	9.7 ± 8.0	86.5 ± 11.9

[a] Ultrafiltration was performed using centrifugal filter units with specified nominal molecular weight limit (NMWL) membranes. [b] Dialyzed inhibitor preparation was used as starting material. Each data point represents mean ± standard deviation of 2 experiments each with 3 replicates

The primary structure of one of the inhibitors was determined and found to have a cysteine motif commonly found in snail and spider toxins (Daquinag and others 1995, 1999). However, the endogenous glycoprotein inhibitor from tobacco horn worm, *Manduca sexta*, was found to have a molecular weight of 380000 on SDS-PAGE gels (Sugumaran and Nellaiappan 2000). In our investigation, considering the system is a

crude mixture, ultrafiltration gave only an estimate of the inhibitor(s) size in house fly pupae and may reflect multiple inhibitors in the extract.

Preliminary Studies on Purification of House Fly Inhibitors

Solvent fractionation

The pupal inhibitor extract prepared by buffer extraction and heat treatment at 70 °C for 30 min as described in Materials and Methods was further subjected to solvent fractionation using cold acetone. Acetone precipitation was performed below 0 °C to prevent protein denaturation caused by temperature increases that occur when organic solvents are mixed with water (Deutscher 1990). The pellet obtained by this technique was suspended in double de-ionized water or 50 mM sodium phosphate buffer (pH 6.0) using a tissue grinder for further analysis. However, a sharp phase separation was observed in the extract upon standing less than 30 min at both refrigerated and room temperatures, regardless of the sample volume.

The acetone extract was then dialyzed overnight against double de-ionized water to remove the residual acetone. After centrifugation, the pellet was immediately suspended in the water or buffer. However, phase separation resumed in a short period of time. The supernatant did not show any inhibition on the enzyme. A similar fractionation method also was performed using cold butanol but the precipitate obtained could not be resolubilized in solution. The overall results indicated that solvent fractionation technique was not a viable step for purification

Ammonium sulfate fractionation and column chromatography

Another attempt to semipurify the house fly inhibitor(s) was ammonium sulfate saturation. Desalted inhibitor extract obtained from 0-95% ammonium sulfate saturation contained 65% of the original activity. The inhibitor extract clarified by centrifugation

was applied to a Sephadex G25-column previously equilibrated with 50 mM sodium phosphate buffer (pH 6.0). The void volume of the column determined using blue dextran was 96 mL. The elution profile is shown in Figure 6-10. The inhibitor activity peak was eluted soon after the void volume. A considerable amount of 280 nm absorbing materials showing no inhibitor activity were eluted from the column just after the inhibitor activity peak (Figure 6-10).

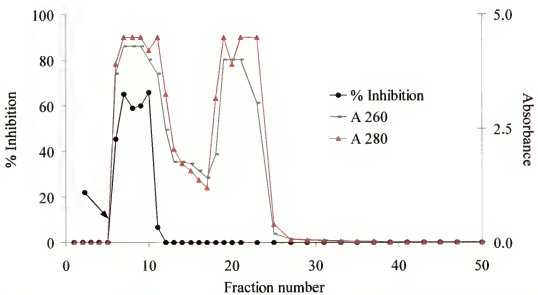


Figure 6-10. Elution profile of inhibitor activity on Sephadex G-25. The arrow indicates the void volume (V_0 = 96 mL) of the column

The active fractions were pooled and chromatographed in a Toyopeal 550C column prequilibrated with 50 mM sodium phosphate buffer (pH 6.0). The unbound materials were removed with 4 column volumes of the same buffer. A considerable amount of 280 nm absorbing materials with no PPO inhibitory activity eluted from the column. Any attempt to show the activity of the potential bound materials using a linear gradient of NaCl was unsuccessful. This was because of the inhibition of apple PPO by sodium chloride. Chloride ion was previously shown as a noncompetitive inhibitor of PPO (Janovitz-Klapp and others 1990). Additionally, the introduction of increasing chloride

concentration into the reaction medium was shown to decrease the pH by modification of the equilibrium constants of the buffer system; and a slight decrease in pH significantly affected the potential activity of apple PPO (Rouet-Mayer and Philippon 1986). Since pH also has a critical effect on the enzyme activity (Figure 6-3), no attempts were made to elute the bound materials from the column by gradually changing the pH of the eluting solution.

The purification studies suggested that the sensitivity of apple PPO activity to the changing composition of the eluting buffer hinders detection of the inhibitory activity of bound materials on the enzyme. However, Tsukamoto and others (1992) demonstrated selective elution of house fly inhibitors for house fly PPO from a cation exchange column with a linear gradient of 0-0.6 M NaCl. The inhibitor activity was eluted at about 0.4-0.5 M NaCl. It appears that house fly PPO differs from apple PPO in that its activity is not affected by sodium chloride or changes in the composition of the eluting buffer.

CHAPTER 7
CONCLUSIONS

Oxalic acid is a selective inhibitor of PPO. Inhibition of PPO by oxalic acid is very likely due to its binding, chelation, and removal of the active-site copper from the enzyme. The pH dependence of inhibition may be of significant value in predicting the effectiveness of an organic dicarboxylic acid and its formulations as an alternative antibrowning agent. However, specific control measures for individual systems would be needed due to the differences in inhibitor response of PPOs from different plant sources, despite the inhibition mechanism being similar.

Immersing banana and apple slices in oxalic acid solutions effectively reduced browning. When relative antibrowning potency of oxalic acid was compared with other dicarboxylic acids (malonic, glutaric and succinic acids), none of the alternative acids was found to be as effective an antibrowning agent as oxalic acid. Moreover, malonic, glutaric and succinic acids did not act as browning inhibitors on apple slices; rather they enhanced browning. Therefore, it was concluded that the ability of oxalic acid to inhibit browning depends on its chemical structure and properties. Additionally, evaluation of temporal changes in color spectra provided additional insight into quantitative changes of color during browning and this method was suggested as an alternative way of determining the effectiveness of a treatment on food color.

Combining oxalic acid with ascorbic acid synergistically enhanced browning inhibition for apple slices but was not as effective for banana. Performance of an

oxalic-ascorbic acid treatment as an antibrowning process depends on the specific food system. A low level of oxalic acid along with other natural inhibitor(s) may have the potential to prevent browning of sliced banana. Apple slices treated with oxalic acid at concentrations well below natural levels retained their color for over 5 h. Using oxalic acid in conjunction with ascorbic acid appears to have potential to control browning of apples. However, additional nutritional studies are required to address the issue of mineral availability if such treatments are to have application in the food industry.

A new type of apple PPO inhibitor(s) was discovered from house fly (*Musca domestica* L.). The crude inhibitor preparation reduced apple PPO activity by 80% at a reaction pH optimal for the enzyme activity. The potential PPO inhibitor(s) was characterized by employing heat, freezing and thawing, irradiation, pH adjustment and ultrafiltration studies. In light of the fact that these results represent the first attempt at identifying inhibitor(s) of the house fly on PPO from higher plants, further studies will be needed to identify the unknown inhibitor(s) and to determine how this inhibitor(s) works on other plant PPOs, and the mechanism by which it works on PPOs. Successful identification of the inhibitor(s) may be a value to food science.

LIST OF REFERENCES

Ahvenainen R. 1996. New approaches in improving the shelf life of minimally processed fruits and vegetables. Trends Food Sci Technol 7: 179-200.

Amorim HW, Melo M. 1991. Significance of enzymes in non-alcoholic coffee beverage. In: Fox PF, editor. Food enzymology. New York: Elsevier Science Publishing. p 189-209.

Andersen SO, Peter MG, Roepstorff P. 1996. Cuticular sclerotization in insects. Comp Biochem Physiol 113B: 689-705.

Andersen SO. 1979. Biochemistry of insect cuticle. Ann Rev Entomol 24: 29-61.

Ashie INA, Simpson BK, Smith JP. 1996. Mechanisms for controlling enzymatic reactions in foods. Crit Rev Food Sci Nutr 36: 1-30.

Bachem CWB, Speckmann G, Linde PCG, Verheggen FTM, Hunt MD, Steffens JC, Zabeau M. 1994. Antisense expression of polyphenol oxidase genes inhibits enzymatic browning in potato tubers. Bio/Technol 12: 1101-1105.

Barrett DM, Lee CY, Liu FW. 1991. Changes in the activity and subcellular distribution of PPO in 'Delicious' apples during controlled atmosphere storage. J Food Biochem 15: 185-199.

Bashan Y, Okon Y, Henis Y. 1987. Peroxidase, polyphenoloxidase, and phenols in relation to resistance against *Pseudomonas syringae* pv. tomato in tomato plants. Can J Bot 65: 366-372.

Becker DK, Dugdale B, Smith MK, Harding RM, Dale JL. 2000. Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv 'Grand Nain' via microprojectile bombardment. Plant Cell Reports 19: 229-234.

Boss PK, Gardner RC, Janssen B and Ross GS. 1995. An apple polyphenol oxidase cDNA is up-regulated in wounded tissues. Plant Mol Biol 27: 429-433.

Bradford MM. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Brunet PCJ. 1980. The metabolism of the aromatic amino acids concerned in the cross-linking of insect cuticle. Insect Biochem 10: 467-500.

Chen L, Mehta A, Berenbaum M, Zangerl AR, Engeseth NJ. 2000. Honeys from different floral sources as inhibitors of enzymatic browning in fruit and vegetable homogenates. J Agric Food Chem 48: 4997-5000.

Chevalier T, Rigal D, Mbeguie-A-Mbeguie D, Gauillard F, Richard-Forget F, Fils-Lycaon BR. 1999. Molecular cloning and characterization of apricot fruit polyphenol oxidase. Plant Physiol 119: 1261-1269.

Cho YK, Ahn HK. 1999. Purification and characterization of polyphenol oxidase from potato: I. Purification and properties. J Food Biochem 23: 577-592.

Coetzer C, Corsini D, Love S, Pavek J, Tumer N. 2001. Control of enzymatic browning in potato (*Solanum tuberosum* L.) by sense and antisense RNA from tomato polyphenol oxidase. J Agric Food Chem 49: 652-657.

Constabel CP, Berger DR, Ryan CA. 1995. Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. Proc Natl Acad Sci USA 92: 407-411.

Cory JG, Frieden E. 1967. Differential reactivities of tyrosine residues of proteins to tyrosinase. Biochem 6: 121-126.

Coseteng MY, Lee CY. 1987. Changes in apple polyphenoloxidase and polyphenol concentrations in relation to degree of browning. J Food Sci 52: 985-989.

Daquinag AC, Sato T, Koda H, Takao T, Fukuda M, Shimonishi Y, Tsukamoto T. 1999. A novel endogenous inhibitor of phenoloxidase from *Musca domestica* has a cysteine motif commonly found in snail and spider toxins. Biochem 38(7): 2179-2188.

Daquinag AC, Nakamura S, Takao T, Shimonishi Y, Tsukamoto T. 1995. Primary structure of a potent endogenous Dopa-containing inhibitor of phenol oxidase from *Musca domestica*. Proc Natl Acad Sci U.S.A. 92(7): 2964-2968.

Das JR, Bhat SG, Gowda LR. 1997. Purification and characterization of a polyphenol oxidase from the Kew cultivar of Indian pineapple fruit. J Agric Food Chem 45: 2031-2035.

Deutscher MP. 1990. Methods in enzymology, Vol 182, guide to protein purification. New York: Academic Press. 894 p.

Dietler C, Lerch K. 1982. Reaction inactivation of tyrosinase. In: King TE, Mason HS, Morrison M, editors. Oxidases and related redox systems. New York: Pergamon Press. p 305-317.

Dixon M, Webb EC. 1979. Enzymes. 3rd ed. New York: Academic Press. 1116 p.

Dry IB, Robinson SP. 1994. Molecular cloning and characterization of grape berry polyphenol oxidase. Plant Mol Biol 26: 495-502.

Duckworth OW, Martin ST. 2001. Surface complexation and dissolution of hematite by C_1-C_6 dicarboxylic acids at pH = 5.0. Geochimica et Cosmochimica Acta 65: 4289-4301.

Engineering and Cyber Solutions. 2003. http://www.eng-n-cybersolutions.com. Visited October 2003.

Escribano J, Cabanes J, Garcia-Carmona F. 1997. Characterization of latent polyphenol oxidase in table beet: Effect of sodium dodecyl sulphate. J Sci Food Agric 73: 34-38.

Eskin NAM. 1990. Biochemistry of food spoilage: Enzymatic browning. In: Eskin NAM, editor. Biochemistry of foods. New York: Academic Press. p 401-432.

Eskin NAM, Henderson HM, Townsend RJ. 1971. Browning reactions in foods. In: Eskin NAM, Henderson HM, Townsend RJ, editors. Biochemistry of foods. New York: Academic Press. p 69-83.

Espin JC, Wichers HJ. 1999. Activation of a latent mushroom (*Agaricus bisporus*) tyrosinase isoform by sodium dodecyl sulfate (SDS). Kinetic properties of the SDS-activated isoform. J Agric Food Chem 47: 3518-3525.

Espin JC, Ochoa M, Tudela J, Garcia-Canovas F. 1997. Monophenolase activity of strawberry polyphenol oxidase. Phytochem 45: 667-670.

Felton GW, Donato KK, Broadway RM, Dufey SS. 1992b. Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a noctuid herbivore, *Spodoptera exigua*. J Insect Physiol 38: 277-285.

Felton GW, Workman J, Duffey SS. 1992a. Avoidance of antinutritive plant defense: Role of midgut pH in Colorado potato beetle. J Chem Ecol 18: 571-583.

Felton GW, Donato K, Del Vecchio RJ, Duffey SS. 1989. Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. J Chem Ecol 15: 2667-2694.

Ferrar PH, Walker JRL. 1996. Inhibition of diphenol oxidases: A comparative study. J Food Biochem 20: 15-30.

Ferrar PH, Walker JRL. 1993. *o*-Diphenol oxidase inhibition-an additional role for oxalic acid in the phytopathogenic arsenal of *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*. Physiol Mol Plant Pathol 43: 415-422.

Ferrer OJ, Koburger JA, Simpson BK, Gleeson RA, Marshall MR. 1989. Phenoloxidase levels in Florida Spiny lobster (*Panulirus argus*): Relationship to season and molting stage. Comp Biochem Physiol 93B: 595-599.

Flurkey WH. 1986. Polyphenoloxidase in higher plants. Plant Physiol 81: 614-618.

Fraignier M, Marques L, Fleuriet A, Macheix J. 1995. Biochemical and immunochemical characteristics of polyphenol oxidase from different fruits of Prunus. J Agric Food Chem 43: 2375-2380.

Frylinck L, Dubery IA, Schabort JC. 1987. Biochemical changes involved in stress response and ripening behavior of γ-irradiated mango fruit. Phytochem 26(3): 681-686.

Fujita S, Saari NB, Maegawa M, Tetsuka T, Hayashi N, Tono T. 1995. Purification and properties of polyphenol oxidase from cabbage (*Brassica oleracea* L.). J Agric Food Chem 43: 1138-1142.

Furia TE. 1964. EDTA in foods: A technical review. Food Technol 18(12): 50-58.

Galeazzi MAM, Sgarbieri VC, Constantinides SM. 1981. Isolation, purification and physicochemical characterization of polyphenoloxidases (PPO) from a Dwarf varity of banana (*Musa cavendishii*, L). J Food Sci 46: 150-155.

Gaykema WPJ, Hol WGJ, Vereijken JM, Soeter NM, Bak HJ, Beintema JJ. 1984. 3.2 A° structure of the copper-containing, oxygen-carrying protein *Panulirus interruptus* haemocyanin. Nature 309: 23-29.

Gillespie JP, Kanost MR, Trenczek T. 1997. Biological mediators of insect immunity. Ann Rev Entomol 42: 611-643.

Golan-Goldhirsh A, Osuga DT, Chen AO, Whitaker JR. 1992. Effect of ascorbic acid and copper on proteins. In: D'Souza VT, Feder J, editors. The bioorganic chemistry of enzymatic catalaysis: An homage to Myron L. Blender. Florida: CRC Press. p 61-76.

Gooding PS, Bird C, Robinson SP. 2001. Molecular cloning and characterization of banana fruit polyphenol oxidase. Planta 213: 748-757.

Guil JL, Torija ME, Gimenez JJ, Rodriguez-Garcia I, Gimenez A. 1996. Oxalic acid and calcium determination in wild edible plants. J Agric Food Chem 44: 1821-1823.

Hara T, Miyoshi T, Tsukamoto T. 1993. Comparative studies on larval and pupal phenoloxidase of the housefly, *Musca domestica* L. Comp Biochem Physiol 106B: 287-292.

Hara T, Tsukamoto T, Watanabe K, Yamasaki N, Funatsu M. 1991. Properties of cuticular phenoloxidase from pupae of the housefly, *Musca domestica* L. Agric Biol Chem 55: 13-17.

Harel E, Mayer AM, Shain Y. 1965. Purification and multiplicity of catechol oxidase from apple chloroplasts. Phytochem 4: 783-790.

Haruta M, Murata M, Kadokura H, Homma S. 1999. Immunological and molecular comparison of polyphenol oxidase in Rosaceae fruit trees. Phytochem 50: 1021-1025.

Haruta M, Murata M, Hiraide A, Kadokura H, Yamasaki M, Sakuta M, Shimizu S, Homma S. 1998. Cloning genomic DNA encoding apple polyphenol oxidase and comparison of the gene product in *Escherichia coli* and in apple. Biosci Biotechnol Biochem 62: 358-362.

Heimdal H, Larsen LM, Poll L. 1994. Characterization of polyphenol oxidase from photosynthetic and vascular lettuce tissues (*Lactuca sativa*). J Agric Food Chem 42: 1428-1433.

Hepburn HR. 1985. The integument. In: Blum MS, editor. Fundamentals of insect physiology. New York: John Wiley & Sons. p 140-183.

Hill HZ. 1992. The function of melanin or six blind people examine an elephant. BioEssays 14: 49-56.

Himmelwright RS, Eickman NC, Lubien CD, Lerch K, Solomon EI. 1980. Chemical and spectroscopic studies of the binuclear copper active site of Neurospora tyrosinase: Comparison to hemocyanins. J Am Chem Soc 102: 7339-7344.

Hogsette JA. 1992. New diets for production of house flies and stable flies (Diptera: Muscidae) in the laboratory. J Econ Entomol 85(6): 2291-2294.

Hopkins TL, Kramer KJ. 1992. Insect cuticle sclerotization. Ann Rev Entomol 37: 273-302.

Huber M, Hintermann G, Lerch K. 1985. Primary structure of tyrosinase from *Streptomyces glaucescens*. Biochem 24: 6038-6044.

Hunt MD, Eannetta NT, Yu H, Newman SM, Steffens JC. 1993. cDNA cloning and expression of potato polyphenol oxidase. Plant Mol Biol 21: 59-68.

Ilarslan H, Palmer RG, Imsande J, Horner HT. 1997. Quantitative determination of calcium oxalate in developing seeds of soybean (Leguminosae). Am J Bot 84: 1042-1046.

Interesse FS, Ruggiero P, D'Avella G, Lamparelli F. 1980. Partial purification and some properties of wheat (*Triticum aestivum*) o-diphenolase. J Sci Food Agric 31: 459-466.

Ito S, Kato T, Shinpo K, Fujita K. 1984. Oxidation of tyrosine residues in proteins by tyrosinase. Biochem J 222: 407-411.

Janovitz-Klapp AH, Richard FC, Goupy PM, Nicolas JJ. 1990. Inhibition studies on apple polyphenol oxidase. J Agric Food Chem 38: 926-931.

Janovitz-Klapp A, Richard F, Nicolas J. 1989. Polyphenoloxidase from apple, partial purification and some properties. Phytochem 28: 2903-2907.

Jayaraman KS, Ramanuja MN, Vijayaraghavan PK, Vaidyanathan CS. 1987. Studies on the purification of banana polyphenoloxidase. Food Chem 24: 203-217.

Jayaraman KS, Ramanuja MN, Dhakne YS, Vijayaraghavan PK. 1982. Enzymatic browning in some banana varieties as related to polyphenoloxidase activity and other endogenous factors. J Food Sci Technol 19(Sept/Oct): 181-186.

Jimenez M, Garcia-Carmona F. 1999. Myricetin, an antioxidant flavonol, is a substrate of polyphenol oxidase. J Sci Food Agric 79: 1993-2000.

Jimenez M, Garcia-Carmona F. 1996. The effect of sodium dodecyl sulphate on polyphenol oxidase. Phytochem 42: 1503-1509.

Kahn V. 1985. Effect of proteins, protein hydrolyzates and amino acids on *o*-dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado, and banana. J Food Sci 50: 111-115.

Kahn V, Andrawis A. 1985. Inhibition of mushroom tyrosinase by tropolone. Phytochem 24: 905-908.

Kahn V, Pomerantz SH. 1980. Monophenolase activity of avocado polyphenol oxidases. Phytochem 19: 379-385.

Kermasha S, Goetghebeur M, Monfette A, Metche M, Rovel B. 1993. Inhibitory effects of cysteine and aromatic acids on tyrosinase activity. Phytochem 34: 349-353.

Kim J, Marshall MR, Wei C. 2000. Polyphenoloxidase. In: Haard NF, Simpson BK, editors. Seafood enzymes utilization and influence on postharvest seafood quality. New York: Marcel Dekker. p 271-315.

Koussevitzky S, Ne'eman E, Sommer A, Steffens JC, Harel E. 1998. Purification and properties of a novel chloroplast stromal peptidase. J Biol Chem 273: 27064-27069.

Kowalski SP, Eannetta NT, Hirzel AT, Steffens JC. 1992. Purification and characterization of polyphenol oxidase from glandular trichomes of *Solanum berthaultii*. Plant Physiol 100: 677-684.

Laidler KJ, Peterman BF. 1983. Temperature effects in enzyme kinetics. In: Purich DL, editor. Contemporary enzyme kinetics and mechanism. New York: Academic Press.p 149-171.

Lanker T, King TG, Arnold SW, Flurkey WH. 1987. Active, inactive and in vitro synthesized forms of polyphenoloxidase during leaf development. Physiol Plant 69: 323-329.

Laveda F, Nunez-Delicado E, Garcia-Carmona F, Sanchez-Ferrer A 2001. Proteolytic activation of latent paraguaya peach PPO. Characterization of monophenolase activity. J Agric Food Chem 49: 1003-1008.

Laveda F, Nunez-Delicado E, Garcia-Carmona F, Sanchez-Ferrer A. 2000. Reversible sodium dodecyl sulfate activation of latent peach polyphenol oxidase by cyclodextrins. Arch Biochem Biophys 379: 1-6.

Lax AR, Vaughn KC. 1991. Colocalization of polyphenol oxidase and photosystem II proteins. Plant Physiol 96: 26-31.

Lax AR, Vaughn KC, Templeton GE. 1984. Nuclear inheritance of polyphenol oxidase in Nicotiana. J Heredity 75: 285-287.

Lee CY, Smith NL, Pennesi AP. 1983. Polyphenoloxidase from DeChaunac grapes. J Sci Food Agric 34: 987-991.

Lehninger AL, Nelson DL, Cox MM. 1993. Principles of biochemistry. 2nd ed. New York: Worth Publishers. 1013 p.

Lerch K. 1995. Tyrosinase: Molecular and active-site structure. In: Lee CY, Whitaker JR, editors. Enzymatic browning and its prevention. ACS Symposium Series 600. Washington D.C.: American Chemical Society. p 64-80.

Lerch K. 1983. Neurospora tyrosinase: structural, spectroscopic and catalytic properties. Mol Cell Biochem 52: 125-138.

Lerner AB. 1953. Metabolism of phenylalanine and tyrosine. Adv Enzymol 14: 73-128.

Lopez AS, Dimick PS. 1991. Enzymes involved in cocoa curing. In: Fox PF, editor. Food enzymology. New York: Elsevier Science Publishing. p 211-236.

Lozano-De-Gonzalez PG, Barrett DM, Wrolstad RE, Durst RW. 1993. Enzymatic browning inhibited in fresh and dried apple rings by pineapple juice. J Food Sci 58: 399-404.

Luo Y, Barbosa-Canovas GV. 1995. Inhibition of apple-slice browning by 4-hexylresorcinol. In: Lee CY, Whitaker JR, editors. ACS Symposium Series 600 Enzymatic Browning and Its Prevention. Washington, DC: American Chemical Society. p 240-250.

Luzuriaga DA. 1999. Application of computer vision and electronic nose technologies for quality assessment of color and odor of shrimp and salmon [Ph.D. thesis]. Gainesville: Univ of Florida. 268 p. Available from: Univ of Florida Library.

Luzuriaga DA, Balaban MO, Yeralan, S. 1997. Analysis of visual quality attributes of white shrimp by machine vision. J Food Sci 62:113-118.

Macheix J, Fleuriet A, Billot J. 1990. Phenolic compounds in fruit processing. In: Macheix J, Fleuriet A, Billot J, editors. Fruit phenolics. Florida: CRC Press. p 239-312.

Marciano P, Lenna PD, Magro P. 1983. Oxalic acid, cell wall-degrading enzymes and pH in pathogenesis and their significance in the virulence of two *Sclerotinia sclerotiorum* isolates on sunflower. Physiol Plant Pathol 22: 339-345.

Marques L, Fleuriet A, Macheix J. 1995. Characterization of multiple forms of polyphenoloxidase from apple fruit. Plant Physiol Biochem 33: 193-200.

Martinez MV, Whitaker JR. 1995. The biochemistry and control of enzymatic browning. Trends Food Sci Technol 6: 195-200.

Mason HS, Peterson EW. 1965. Melanoproteins. I. Reactions between enzyme-generated quinones and amino acids. Biochim Biophys Acta 111: 134-146.

Mason HS. 1957. Mechanisms of oxygen metabolism. Adv Enzymol 19: 79-231.

Mason HS. 1956. Structures and functions of the phenolase complex. Nature 177: 79-81.

Matheis G, Whitaker JR. 1984. Modification of proteins by polyphenol oxidase and peroxidase and their products. J Food Biochem 8: 137-162.

Mathew AG, Parpia HAB. 1971. Food browning as a polyphenol reaction. Adv Food Res 19: 75-145.

Mayer AM, Harel E. 1991. Phenoloxidases and their significance in fruit and vegetables. In: Fox PF, editor. Food enzymology. New York: Elsevier Science Publishing. p 373-398.

Mayer AM, Harel E. 1979. Polyphenol oxidases in plants. Phytochem 18: 193-215.

McEvily AJ, Iyengar R, Otwell W.S. 1992. Inhibition of enzymatic browning in foods and beverages. Crit Rev Food Sci Nutr 32: 253-273.

Miller AR, Kelley TJ, Mujer CV. 1990. Anodic peroxidase isoenzymes and polyphenol oxidase activity from cucumber fruit: tissue and substrate specificity. Phytochem 29: 705-709.

Miller DD. 1996. Principles of mineral chemistry. In: Fennema OR, editor. Food chemistry. New York: Marcel Dekker. p 618-625.

Molnar-Perl I, Friedman M. 1990. Inhibition of browning by sulfur amino acids. 2. Fruit juices and protein-containing foods. J Agric Food Chem. 38: 1648-1651.

Montgomery MW, Sgarbieri VC. 1975. Isoenzymes of banana polyphenol oxidase. Phytochem 14: 1245-1249.

Moore BM, Flurkey WH. 1990. Sodium dodecyl sulfate activation of a plant polyphenoloxidase. Effect of sodium dodecyl sulfate on enzymatic and physical characteristics of purified broad bean polyphenoloxidase. J Biol Chem 265: 4982-4988.

Morgan PB, Smittle BJ, Patterson RS. 1986. Use of irradiated pupae to mass-culture the microhymenopterous pupal parasitoid *Spalangia endius* Walker (Hymenoptera: Pteromalidae). I. *Musca domestica* L. (Diptera: Muscidae). J Entomol Sci 21(3): 222-227.

Murata M, Haruta M, Murai N, Tanikawa N, Nishimura M, Homma S, Itoh Y. 2000. Transgenic apple (*Malus* x *domestica*) shoot showing low browning potential. J Agric Food Chem 48: 5243-5248.

Murata M, Tsurutani M, Hagiwara S, Homma S. 1997. Subcellular location of polyphenol oxidase in apples. Biosci Biotech Biochem 61: 1495-1499.

Murata M, Tsurutani M, Tomita M, Homma S, Kaneko K. 1995. Relationship between apple ripening and browning: Changes in polyphenol content and polyphenol oxidase. J Agric Food Chem 43: 1115-1121.

Nation JL, Smittle BJ, Milne K, Dykstra TM. 1995. Influence of irradiation on development of Caribbean fruit fly (Diptera: Tephritidae) larvae. Ann Entomol Soc Am 88(3): 348-352.

Nicolas JJ, Richard-Forget FC, Goupy PM, Amiot M, Aubert SY. 1994. Enzymatic browning reactions in apple and apple products. Crit Rev Food Sci Nutr 34: 109-157.

Oba K, Iwatsuki N, Uritani I, Alvarez AM, Garcia VV. 1992. Partial purification and characterization of polyphenol oxidase isozymes in banana bud. Biosci Biotech Biochem 56: 1027-1030.

Oktay M, Kufrevioglu I, Kocacaliskan I, Sakiroglu H. 1995. Polyphenoloxidase from Amasya apple. J Food Sci 60: 494-496.

Osuga D, Van Der Schaaf A, Whitaker JR. 1994. Control of polyphenol oxidase activity using a catalytic mechanism. In: Yada RY, Jackman RL, Smith JL, editors. Protein structure-function relationships in foods. New York: Chapman and Hall. p 62-88.

Oszmianski J, Lee CY. 1990. Inhibition of polyphenol oxidase activity and browning by honey. J Agric Food Chem 38: 1892-1895.

Park EY, Luh BS. 1985. Polyphenol oxidase of kiwifruit. J Food Sci 50: 678-684.

Park HJ. 1999. Development of advanced edible coatings for fruits. Trends Food Sci Technol 10: 254-260

Perez-Gilabert M, Carmona FG. 2000. Characterization of catecholase and cresolase activities of eggplant polyphenol oxidase. J Agric Food Chem 48: 695-700.

Pierpoint WS. 1966. The enzymatic oxidation of chlorogenic acid and some reactions of the quinone produced. Biochem J 98: 567-580.

Pifferi PG, Baldassari L, Cultrera R. 1974. Inhibition by carboxylic acids of an *o*-diphenol oxidase from *Prunus avium* fruits. J Sci Food Agric 25:263-270.

Prenen JAC, Boer P, Mees EJD. 1984. Absorption kinetics of oxalate from oxalate-rich food in man. Am J Clin Nutr 40: 1007-1010.

Pye AE. 1974. Microbial activation of prophenoloxidase from immune insect larvae. Nature 251: 610-613.

Rathjen AH, Robinson SP. 1992. Aberrant processing of polyphenol oxidase in a variegated grapevine mutant. Plant Physiol 99: 1619-1625.

Ray H, Hammerschmidt R. 1998. Responses of potato tuber to infection by Fusarium sambucinum. Physiol Mol Plant Pathol 53: 81-92.

Richards AG. 1978. The chemistry of insect cuticle. In: Rockstein M, editor. Biochemistry of insects. New York: Academic Press. p 205-231.

Robb DA, Swain T, Mapson LW. 1966. Substrates and inhibitors of the activated tyrosinase of broad bean (Vicia faba L.). Phytochem 5: 665-675.

Robinson SP, Loveys BR, Chacko EK. 1993. Polyphenol oxidase enzymes in the sap and skin of mango fruit. Aust J Plant Physiol 20: 99-107.

Rouet-Mayer M, Philippon J. 1986. Inhibition of catechol oxidases from apples by sodium chloride. Phytochem 25: 2717-2719.

Sanchez-Ferrer A, Rodriguez-Lopez JN, Garcia-Canovas F, Garicia-Carmona F. 1995. Tyrosinase: a comprehensive review of its mechanism. Biochim Biophys Acta 1247: 1-11.

Sanchez-Ferrer A, Laveda F, Garcia-Carmona F. 1993. Cresolase activity of potato tuber partially purified in a two-phase partition system. J Agric Food Chem 41: 1225-1228.

Sapers GM. 1993. Browning of foods: Control by sulfites, antioxidants, and other means. Food Technol 47(10): 75-84.

Sapers GM, Miller RL. 1993. Control of enzymatic browning in pre-peeled potatoes by surface digestion. J Food Sci 58:1076-1078.

Sapers GM, Hicks KB, Phillips JG, Garzarella L, Pondish DL, Matulaitis RM, McCormack TJ, Sondey SM, Seib PA, Ei-Atawy YS. 1989. Control of enzymatic browning in apple with ascorbic acid derivatives, polypheol oxidase inhibitors, and complexing agents. J Food Sci 54: 997-1012.

Sapers GM, Douglas FW. 1987. Measurement of enzymatic browning at cut surfaces and in juice of raw apple and pear fruits. J Food Sci 52:1258-1262.

Sato M. 1980a. Inhibition by oxalates of spinach chloroplast phenolase in unfrozen and frozen states. Phytochem 19: 1613-1617.

Sato M. 1980b. Reactivation by copper of phenolase pre-inactivated by oxalate. Phytochem 19: 1931-1933.

Serradell MDLA, Rozenfeld PA, Martinez GA, Civello PM, Chaves AR, Anon MC. 2000. Polyphenoloxidase activity from strawberry fruit (*Fragaria* x *ananassa*, Duch., cv Selva) characterization and partial purification. J Sci Food Agric 80: 1421-1427.

Shahar T, Henning N, Gutfinger T, Hareven D, Lifschitz E. 1992. The tomato 66.3 kD polyphenoloxidase gene: Molecular identification and developmental expression. The Plant Cell 4: 135-147.

Sherman TD, Gardeur TL, Lax AR. 1995. Implications of the phylogenetic distribution of polyphenol oxidase in plants. . In: Lee CY, Whitaker JR, editors. Enzymatic browning and its prevention. ACS Symposium Series 600. Washington D.C.: American Chemical Society. p 103-119.

Sherman TD, Vaughn KC, Duke SO. 1991. A limited survey of the phylogenetic distribution of polyphenol oxidase. Phytochem 30: 2499-2506.

Sheptovitsky YG, Brudvig GW. 1996. Isolation and characterization of spinach photosystem II membrane-associated catalase and polyphenol oxidase. Biochem 35: 16255-16263.

Simpson BK, Marshall MR, Otwell WS. 1988. Phenoloxidases from pink and white shrimp: Kinetic and other properties. J Food Biochem 12: 205-217.

Solomon EI, Lowery MD. 1993. Electronic structure contributions to function in bioinorganic chemistry. Science 259: 1575-1581.

Solomon EI, Baldwin MJ, Lowery MD. 1992. Electronic structures of active sites in copper proteins: Contributions to reactivity. Chem Rev 92: 521-542.

Sommer A, Ne' Eman E, Steffens JC, Mayer AM, Harel E. 1994. Import, targeting and processing of a plant polyphenol oxidase. Plant Physiol 105: 1301-1311.

Son SM, Moon KD, Lee CY. 2001. Inhibitory effects of various antibrowning agents on apple slices. Food Chem 73: 23-30.

Son SM, Moon KD, Lee CY. 2000a. Kinetic study of oxalic acid inhibition on enzymatic browning. J Agric Food Chem 48: 2071-2074.

Son SM, Moon KD, Lee CY. 2000b. Rhubarb juice as a natural antibrowning agent. J Food Sci 65: 1288-1289.

Steffens JC, Harel E, Hunt MD. 1994. Polyphenol oxidase. In: Ellis BE, Kuroki GW, Stafford HA, editors. Recent advances in phytochemistry, Vol. 28, Genetic engineering of plant secondary metabolism. New York: Plenum Press.p 275-312.

Stelzig DA, Akhtar S, Ribeiro S. 1972. Catechol oxidase of Red Delicious apple peel. Phytochem 11: 535-539.

Sugumaran M, Nellaiappan K. 2000. Characterization of a new phenoloxidase inhibitor from the cuticle of *Manduca Sexta*. Biochem Biophys Res Commun 268(2): 379-383.

Sugumaran M. 1998. Unified mechanism for sclerotization of insect cuticle. Adv Insect Physiol 27: 229-334.

Tan BK, Harris ND. 1995. Maillard reaction products inhibit apple polyphenoloxidase. Food Chem 53: 267-273.

Thipyapong P, Steffens JC. 1997. Tomato polyphenol oxidase. Differential response of the polyphenol oxidase F promoter to injuries and wound signals. Plant Physiol 115: 409-418.

Thipyapong P, Hunt MD, Steffens JC. 1995. Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase. Phytochem 40: 673-676.

Thomas P, Janave MT. 1986. Isoelectric focusing evidence for banana isoenzymes with mono and diphenolase activity. J Food Sci 51: 384-387.

Thygesen PW, Dry IB, Robinson SP. 1995. Polyphenol oxidase in potato. A multigene family that exhibits differential expression patterns. Plant Physiol 109: 525-531.

Tipton KF, Dixon HBF. 1983. Effects of pH on enzymes. In: Purich DL, editor. Contemporary enzyme kinetics and mechanism. New York: Academic Press. p 97-148.

Tsukamoto T, Ichimaru Y, Kanegae N, Watanabe K, Yamaura I, Katsura Y, Funatsu M. 1992. Identification and isolation of endogenous insect phenoloxidase inhibitors. Biochem Biophys Res Commun 184(1): 86-92.

Ullah MR. 1991. Tea. In: Fox PF, editor. Food enzymology. New York: Elsevier Science Publishing. p 163-187.

USDA 1984. Composition of foods: Vegetables and vegetable products. U.S. Dept. of Agriculture Handbook Number 8-11.

Valero E, Garcia-Carmona F. 1998. pH-dependent effect of sodium chloride on latent grape polyphenol oxidase. J Agric Food Chem 46: 2447-2451.

Valero E, Garcia-Carmona F. 1992. Hysteresis and cooperative behavior of a latent plant polyphenoloxidase. Plant Physiol 98: 774-776.

Vamos-Vigyazo L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit Rev Food Sci Nutr 15: 49-127.

Van Gelder CWG, Flurkey WH, Wichers HJ. 1997. Sequence and structural features of plant and fungal tyrosinases. Phytochem 45: 1309-1323.

Vaughn KC, Lax AR, Duke SO. 1988. Polyphenol oxidase: The chloroplast oxidase with no established function. Physiol Plant 72: 659-665.

Vaughn KC, Duke SO. 1984. Function of polyphenol oxidase in higher plants. Physiol Plant 60: 106-112.

Wesche-Ebeling P, Montgomery MW. 1990. Strawberry polyphenoloxidase: Extraction and partial characterization. J Food Sci 55: 1320-1324.

West LS. 1951. The housefly: Its natural history, medical importance, and control. New York: Comstock Publishing Company, associated with Cornell University Press. 584 p.

Whitaker JR. 1995. Polyphenol oxidase. In: Wong DWS, editor. Food enzymes and mechanism. New York: Chapman Hall. P 271-307.

Whitaker JR, Lee CY. 1995. Recent advances in chemistry of enzymatic browning: An overview. In: Lee CY, Whitaker JR, editors. Enzymatic browning and its prevention. ACS Symposium Series 600. Washington D.C.: American Chemical Society. p 2-7.

Whitaker JR. 1994. Principles of enzymology for the food sciences. 2nd ed. New York: Marcel Dekker. 625 p.

Wilcox DE, Porras AG, Hwang YT, Lerch K, Winkler ME, Solomon EI. 1985. Substrate analogue binding to the coupled binuclear copper active site in tyrosinase. J Am Chem Soc 107: 4015-4027.

Yalovsky S, Paulsen H, Michaeli D, Chitnis PR, NechushtaI R. 1992. Involvement of a chloroplast HSP70 heat shock protein in the integration of a protein (light-harvesting complex protein precursor) into the thylakoid membrane. Proc Natl Acad Sci USA 89: 5616-5619.

Yamaura I, Yonekura M, Katsura Y, Ishiguro M, Funatsu M. 1980. Purification and some physico-chemical properties of phenoloxidase from the larvae of housefly. Agric Biol Chem 44: 55-59.

Yang C, Fujita S, Kohno K, Kusubayashi A, Ashrafuzzaman MD, Hayashi N. 2001. Partial purification and characterization of polyphenol oxidase from banana (*Musa sapientum* L.) peel. J Agric Food Chem 49: 1446-1449.

Yemenicioglu A, Ozkan M, Cemeroglu B. 1997. Heat inactivation kinetics of apple polyphenoloxidase and of its latent form. J Food Sci 62: 508-510.

Yong G, Leone C, Strothkamp KG. 1990. *Agaricus bisporus* metapotyrosinase: Preparation, characterization, and conversion to mixed-metal derivatives of the binuclear site. Biochem 29: 9684-9690.

Yoruk R, Marshall MR. 2003. Physicochemical properties and function of plant polyphenol oxidase: A review. J Food Biochem 27: 361-422.

Zawistowski J, Biliaderis CG, Eskin NAM. 1991. Polyphenol oxidase. In: Robinson DS, Eskin NAM, editors. Oxidative enzymes in foods. New York: Elsevier. p 217-273.

Zhou P, Smith NL, Lee CY. 1993. Potential purification and some properties of Monroe apple peel polyphenol oxidase. J Agric Food Chem 41: 532-536.

BIOGRAPHICAL SKETCH

Ruhiye Yoruk received her B.S degree in biology from Anadolu University, Eskisehir, Turkey, in 1992. After graduation, she entered the masters program in molecular biology at Anadolu University and completed her graduate coursework. In 1994, she received a full Academic Scholarship from the Turkish Ministry of National Education to study abroad. In fall 1995, she began the masters program in the Department of Biological, Chemical, and Physical Sciences at Illinois Institute of Technology in Chicago, Illinois and completed her M.S. in biology in fall 1997. Because of her interest in food chemistry and biochemistry, Ruhiye began the doctoral program in the Department of Food Science and Human Nutrition, University of Florida in spring 1998. Her research has focused on the prevention of adverse enzymatic browning proceeding in several fruits and vegetables during handling and processing. She worked on a collaborative university-Food and Agricultural Organization of the UN and university-USDA project on inhibition of apple PPO activity using natural inhibitor(s) from housefly. As she continued to work in this area, she gave particular attention to the relative antibrowning potency of the natural compound, oxalic acid, on fresh-cut surfaces of banana and apple. She also served as a teaching assistant under the supervision of her peer mentor, Dr. Marshall, during her doctoral studies. She received her Ph.D. in fall 2003 and will continue working in a research and educational institution.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maurice R. Marshall, Chair
Professor of Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Murat O. Balaban
Professor of Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jesse F. Gregory
Professor of Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James F. Preston
Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jerome A. Hogsette
Assistant Professor of Entomology and Nematology

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 2003

Dean, College of Agricultural and Life Sciences

Dean, Graduate School